MOLECULAR DETECTION OF RIFAMPICIN RESISTANCE IN MYCOBACTERIUM TUBERCULOSIS

DISSERTATION

SUBMITTED FOR

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CERTIFICATE

This is to certify that the dissertation work entitled **"Molecular detection of Rifampicin resistance in Mycobacterium tuberculosis"** submitted by Dr. Priya Santharam, is work done by her during the period of study in this department from June 2007 to February 2010. This work was done under the guidance of Dr. B Appalaraju , Professor and Head, Department of Microbiology, PSG IMS & R.

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CONTENTS

INTRODUCTION

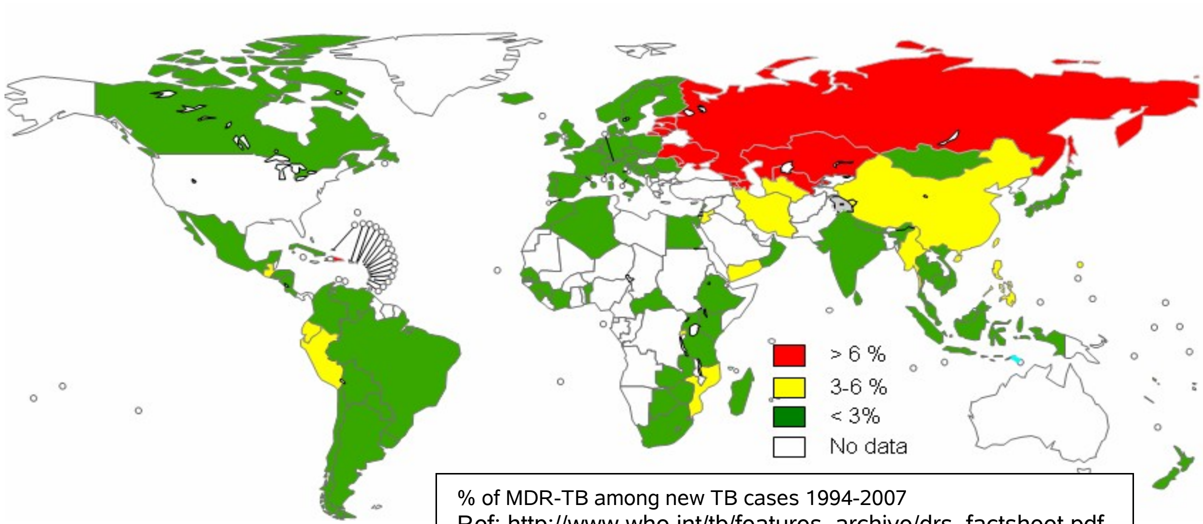
Tuberculosis (TB) is as old as the mankind. TB is the most common cause of death due to a single infectious agent worldwide in adults. According to the recent estimates, one third of the human population (2 billion people) is infected with *Mycobacterium tuberculosis* worldwide[1]. Of these, more than half the cases occur in five South-East Asian countries. India accounts for nearly one third of the global TB burden. In 2007, nearly 1.77 million people died of TB and the global case fatality rate is estimated to be 45% in HIV-positive TB-patients and 35% in HIV-negative TB-patients. Nearly 500,000 people die from TB in India every year — more than 1,000 every day, 1 every minute[2]

The disease though known since ancient times, the organism causing TB was described only a century ago by Robert Koch on 24th March 1882. Until middle of the 20th century, there was no definitive treatment available for TB. With the availability of Streptomycin, Isoniazid and Para aminosalicylic acid (PAS), in the mid 1940s, predictable, curative treatment for TB became a reality. The introduction of Rifampicin, Pyrazinamide and Ethambutol in the subsequent years ushered in the era of short-course treatment. Further, the fully supervised sanatorium based treatment of the earlier days also gave way to the totally unsupervised domiciliary treatment. Soon, it was felt that TB could be easily contained and possibly eradicated. The advent of HIV infection, the acquired immunodeficiency syndrome (AIDS) pandemic in the 1980s, struck a blow to this optimism and there has been a global resurgence of TB especially the multi drug resistant strains. .The rise in tuberculosis (TB) incidence over the last two decades is TB deaths in HIV-infected patients and partly due to the emergence of multidrug resistant strains of the bacteria[3].

Multidrug-resistant tuberculosis (MDR-TB) is among the most worrisome elements of the pandemic of antibiotic resistance because TB patients that fail treatment have a high risk of death[4].

Though studies published from the developing world suggested that drug resistance was a potential problem, it was the emergence of MDR-TB in USA in the 1990s which attracted the attention. A review by WHO of a series of 63 surveys of drug resistant TB carried out between 1985 and1994 led to the conclusion that the problem of drug resistance was global. The WHO, for the first time, introduced the term MDR 'hotspot' where high prevalence of MDR had been observed. The 'hotspot' referred to countries or regions where the combined prevalence of MDR TB exceeded 5%[5].According to a WHO data from 81 countries and 91,577 patients collected between 2002-2006 shows MDR-TB, on average, in 5.3% of all TB cases [6]

In India, prevalence of primary MDR-TB in newly diagnosed cases has been observed to be 3.4 per cent or less. Data meticulously collected at the Tuberculosis Research Centre (TRC), Chennai over the last three decades suggest that MDR-TB levels in newly diagnosed patients have been one per cent or less[7]. Prevalence of MDR-TB among previously treated patients has been observed to be higher.



% of MDR-TB among new TB cases 1994-2007
Ref: http://www.who.int/tb/features_archive/drs_factsheet.pdf

In view of the emerging resistant mutants in the global and Indian scenario, the need for prompt detection of *Mycobacterium tuberculosis* in suspected cases, susceptibility pattern and genetic basis of drug resistance becomes paramount importance. In most laboratories in developing countries, smear examination and culture remains the only available choice. Though culture is the gold standard, it is time consuming due to the long replication time of mycobacteria and results are available only by 4-8 weeks.

Good, reliable laboratory support is seldom available in developing nations. When facilities for culture and sensitivity testing are not available, therapeutic decisions are most often made by Standard Guidelines such as those published by the WHO.

Although the DOTS strategy is the basis of good TB control, the strategy should be modified in some settings to identify drug-resistant cases sooner, and to make use of second-line drugs in appropriate treatment regimens[8].

Aims & Objectives

AIM

- To detect multidrug resistance in *Mycobacterium tuberculosis*

OBJECTIVES

- Isolation and characterization of *Mycobacterium tuberculosis* in respiratory specimens collected from patients attending the RNTCP outpatient clinic and from inpatients at PSG Hospitals by conventional methods.

- To detect drug resistance for Isoniazid and Rifampicin by two phenotypic methods namely Absolute concentration method and MTT assay

- To detect mutations in rpoB gene from the above Rifampicin resistant isolates of *Mycobacterium tuberculosis* by genotypic method using PCR and DNA Sequencing

Review of Literature

LITERATURE REVIEW

Tuberculosis in India is one of the largest public health problems of immense consequence, with an estimated one death per minute[2]. Tuberculosis (TB) has been declared as a global health emergency by the World Health Organization (WHO). This has been mainly due to the emergence of multiple drug resistant strains and the synergy between tubercle bacilli and the human immunodeficiency virus (HIV)[8]

History[9]

Tuberculosis is a disease of great antiquity and has almost certainly caused more suffering and death than any other infection. The clinical features of both respiratory and spinal tuberculosis were well described by Hippocrates in about 4000 BC; accounts of the disease appeared in the Vedas and other ancient Hindu texts, in which it was termed rajayakshma – the king of diseases – and it afflicted Neolithic and pre-Columbian Ameridians.

The transmissible nature of tuberculosis was established by Villemin in 1868 by inoculating rabbits with tuberculous material from humans and cattle. Villemin also established that scrofula (tuberculous lymphadenitis) and pulmonary tuberculosis were manifestations of the same disease process. In 1882 Robert Koch succeeded in cultivating the bacillus on inspissated serum and transmitted the disease to many animals of different species by inoculating with pure cultures of the bacillus. In addition to cultivating the organism, Koch succeeded in staining it by treatment with an alkaline solution of methylene blue for 24 hrs. Subsequently Ehrlich improved the technique by using a hot solution of carbol fuchsin and it is this technique modified by Ziehl and Neelsen that is widely used today.

General Characteristics[9]

Tuberculosis in humans was shown to be caused by two types of bacillus – human and the bovine types designated as *Mycobacterium tuberculosis* and *Mycobacterium bovis* respectively. The mycobacterium tuberculosis complex includes *M.tuberculosis, M.bovis, M.africanum, M.microti and M.canettii.*

The other environmental mycobacteria recognised as causative agents of human disease were called as non tuberculous mycobacteria (NTM) or Mycobacteria other than tubercle bacilli (MOTT)

Mycobacterium tuberculosis.

M. tuberculosis is the most common pathogenic mycobacterial species in the *M.tuberculosis* complex

Scientific classification

Kingdom	:	Bacteria
Phylum	:	Actinobacteria
Order	:	Actinomycetales
Suborder	:	Corynebacterineae
Family	:	Mycobacteriaceae
Genus	:	*Mycobacterium*
Species	:	*tuberculosis*

Habitat

Mycobacterium species are isolated from a wide variety of environmental sources including water, soil, dust and Sphagnum vegetation. Mycobacteria have been classified as pathogens, or free living saprophytes. Obligate pathogens are species that do not appear to multiply outside their hosts and include *M.tuberculosis* complex, *Mycobacterium leprae, Mycobacterium marinum,* and *Mycobacterium paratuberculosis.* Tuberculosis manifests both as pulmonary and extra pulmonary in humans and warm blooded animals and has become a new threat in developing countries in the most recent past[10]**.**

Morphology

M. tuberculosis is a straight or slightly curved rod, about 3µm x 0.3 µm, occurring singly, in pairs or as small clumps. The size depends on conditions of growth, and long filamentous, club shaped and branching forms may be sometimes seen. *Mycobacterium bovis* is usually straighter, shorter and stouter.

Tubercle bacilli have been described as Gram positive, though strictly speaking this is not correct, as after staining with basic dyes they resist decolourisation by alcohol even without the mordanting effect of iodine. When stained with carbol fuchsin by the Ziehl-Neelsen method or by fluorescent dyes (auramine O, rhodamine), they resist decolourisation by 20 percent sulphuric acid and absolute alcohol. This acid and alcohol fastness has been ascribed variously to the presence in the bacillus of an unsaponifiable wax (mycoloic acid) or to a semipermeable membrane around the cell. Electron micrographs of thin sections show that the thick cell wall is composed of three layers enclosing a trilaminar plasma membrane.

Cultural Characteristics[11]

The bacilli grow slowly, the generation time in vitro being 14-15 hours. Colonies appear in about two weeks and may sometimes take up to eight weeks. Optimum temperature is 37ºC and Optimum pH is 6.4-7.0. *M. tuberculosis* is an obligate aerobe. It grows luxuriantly in culture as compared to *Mycobacterium bovis* which grows sparsely. They are termed as eugenic and dysgenic respectively. The addition of 0.5% glycerol improves the growth of *M.tuberculosis.*

Tubercle bacilli do not have exacting growth requirements but are highly susceptible even to traces of toxic substances like fatty acids in culture media. The toxicity is neutralized by serum albumin (or) charcoal. Koch originally grew the bacillus on heat coagulated bovine serum. Several media, both solid and liquid media have been described for the cultivation of tubercle bacilli. The solid media contain egg (Lowenstein-Jensen, Petragnini, Dorset), Blood (Tarshis), serum (Loeffler) or potato (Pawlowsky). The solid medium most widely employed for routine culture is Lowenstein-Jensen (LJ) medium without starch, as recommended by the International Union Against Tuberculosis (IUAT). Among the several liquid media described, Dubo’s, Middle brook’s, Proskauer and beck’s, Sula’s and Sauton’s media are the more common.

On solid media, *M.tuberculosis* forms dry, rough, raised, irregular colonies with a wrinkled surface. They are creamy white, becoming buff coloured on further incubation. In liquid media without dispersing agents the growth begins at the bottom, creeps up the sides and forms a prominent surface pellicle which may extended along the sides above the medium.

Identification of Bacteria

Once an isolate has been recovered in the mycobacteriology laboratory, certain characteristics may be used to classify the isolate before performing biochemical tests.

The first step is to confirm that the isolate recovered in broth or solid media culture is an acid-fast organism by performing an acid-fast stain. Then once the organisms are growing on solid media, phenotypic characteristics such as colony morphology, growth rate, optimum temperature and photo reactivity helps to speciate Mycobacteria. These characteristic do not allow for definitive identification but are presumptive and help in the selection of others, more definitive tests.

Colony Morphology

Colonies of Mycobacteria are generally distinguished as having either a smooth and soft or a rough and friable appearance. Colonies of *M. tuberculosis* that are rough often also exhibit a prominent patterned texture in liquid cultures referred to as cording (curved strands of bacilli)

Growth Rate

Growth rate and recovery time depend on the species of Mycobacteria but are also influenced by media, the incubation temperature, and the initial inoculum size. Mycobacteria are generally categorized as having visible growth within or more than 7 days. Rapid growers are able to produce colonies in fewer than 7 days.

Temperature

The optimum temperature and range at which a Mycobacterial species may grow may be extremely narrow, especially at the time of initial incubation. The growth of *M.tuberculosis* occurs at 37 °C.

Photo-reactivity

Mycobacterium species have traditionally been categorized in three groups according to their photo reactivity characteristics.

Photochromogens - that produce carotene pigment upon exposure to light (color ranges from pale yellow to orange).

Scotochomogens - that produce pigment in the light or the dark.

Non Chromogenic Or Nonphotochromogenic- colonies are buff colored and are nonphotoreactive i.e. on exposure to light does not produce pigment e.g*., M. Tuberculosis*[11]

Biochemical Identification[12]

A panel of biochemical tests is available for the identification of Mycobacterium species. Because Mycobacterium species may show only quantitative differences in enzymes used in biochemical identification, no single biochemical test should be relied on for the identification of a species, for expediency, all necessary biochemical tests should be set up at one time.

1. Niacin test:-

Most Mycobacteria possess the enzyme that converts free niacin to niacin ribonucleotide. Accumulation of niacin detected as nicotinic acid, is the most commonly used biochemical test for the identification of *M. tuberculosis*. Nicotinic acid reacts with cyanogen bromide in the presence of an amine to form a yellow-pigmented compound. This test is positive with *M.tuberculosis* and negative with *M. bovis*.

2. Neutral red test:-

Virulent strains of tubercle bacilli are able to bind neutral red in alkaline buffer solution, while avirulent strains are unable to do so

3. Catalase-peroxidase test:-

Catalase is an intracellular enzyme that can split hydrogen peroxidase into water and oxygen. Catalase in atypical mycobacteria is stable when heated in a suspension to 68ºC while *M.tuberculosis* loses their catalase activity. These help in differentiating tubercle bacilli from atypical Mycobacteria and provide an indication of the sensitivity of the strains to isoniazid. On the other hand, tubercle bacilli are peroxidase positive, but not atypical Mycobacteria. Catalase and peroxidase activities are lost when tubercle bacilli become Isoniazid resistant.

4. Nitrate reduction test:-

The production of nitroreductase, which catalyzes the reduction of nitrate to nitrite, a red color forms by the addition of sulfanilamide and N- naphthylediamine to bacterial suspension incubated in nitrate broth. This is positive with *M.tuberculosis* and negative with *M.bovis*.

5. Growth in the presence of p-nitrobenzoic acid:-

This compound inhibits the growth *M.tuberculosis* complex namely *M. tuberculosis, M.bovis, M.africanum and M.microti* while atypical mycobacteria are resistant and grow well in LJ containing PNB

6. Pyrazinamidase test:-

Pyrazinamidase is an enzyme that hydrolyzes pyrazinamidase to pyrazinoic acid and ammonia. Ferrous ammonium sulfate combines with pyrazinoic acid producing a red pigment. This reaction occurs in about four days and may be useful in distinguishing *Mycobacterium bovis* from *M.tuberculosis* and *M. marinum* from *M. kansasi*

7. Growth in the presence of thiophen-2-carboxylic acid hydrazide:-

This test is useful to distinguish *M.tuberculosis*, which grows in the presence of this compound from other members of the *M. tuberculosis* complex

PATHOGENESIS

Mode of transmission

M. tuberculosis is most commonly transmitted from a patient with infectious pulmonary tuberculosis to other persons by droplet nuclei, which are aerosolized by coughing, sneezing, or speaking.

Signs and symptoms

- Fever and sweating
- Weight loss
- hemoptysis

- Dyspnea
- Thoracic pain
- Hoarseness
- Hepatosplenomegaly, in case of disseminated forms.

Clinical Manifestations

Tuberculosis is classified as pulmonary or extrapulmonary. Before the recognition of HIV infection, 80% of all cases of tuberculosis were limited to the lungs. However, up to two thirds of HIV-infected patients with tuberculosis may have either pulmonary and extra pulmonary disease or extra pulmonary disease alone.

Pulmonary Tuberculosis

Pulmonary tuberculosis can be categorized as primary or post primary (secondary).

Primary Disease

Primary pulmonary tuberculosis results from an initial infection with tubercle bacilli localized to the middle and lower lung zones.

In the majority of cases, the lesion heals spontaneously and may later be evident as a small calcified nodule (Ghon lesion). In children and in persons with impaired immunity (e.g., those with malnutrition or HIV infection), primary pulmonary tuberculosis may progress rapidly to clinical illness. The initial lesion increases in size and can evolve in different ways. In severe cases, the primary site rapidly enlarges, undergoes necrosis, and acute cavitation develops (progressive primary tuberculosis). Enlarged lymph nodes of hilum and mediastinum may compress bronchi, causing obstruction and subsequent segmental or lobar collapse. Partial obstruction may cause obstructive emphysema, and bronchiectasis. Hematogenous dissemination, which is common and is often asymptomatic, may result in the most severe manifestations of primary *M. tuberculosis* infection. Although healing frequently takes place, immunocompromised persons (e.g., patients with HIV infection) may develop miliary tuberculosis and/or tuberculous meningitis.

Post Primary Disease

Post primary disease also called adult-type, reactivation, or secondary tuberculosis, results from endogenous reactivation of latent infection and is usually localized to the apical and posterior segments of the upper lobes, where the high oxygen concentration favours Mycobacterial growth. With cavity formation, liquefied necrotic contents are ultimately discharged into the airways, resulting in satellite lesions within the lungs that may in turn undergo cavitation. Massive involvement of pulmonary segments or lobes produces tuberculous pneumonia. One-third of untreated patients reportedly succumb to severe pulmonary tuberculosis within a few weeks or months after onset.

Physical findings are of limited use in pulmonary tuberculosis. Many patients have no abnormalities detectable by chest examination, while others have detectable rales in the involved areas during inspiration, especially after coughing. The most common hematologic findings are mild anaemia and leukocytosis

Extra Pulmonary Tuberculosis

Tuberculosis can involve any organ system in the body. While pulmonary tuberculosis is the most common presentation, extra pulmonary tuberculosis (EPTB) is also an important clinical problem. The term EPTB has been used to describe isolated occurrence of tuberculosis at body sites other than the lung[13]

Different forms of extra pulmonary tuberculosis includes miliary tuberculosis, neurological tuberculosis, lymph node tuberculosis, tuberculosis pleural effusion, abdominal tuberculosis, genitourinary tuberculosis, ocular tuberculosis, renal tuberculosis, pericardial tuberculosis, pancreatitis, otitis, bone and joint tuberculosis,.

TREATMENT

The treatment of tuberculosis (TB) has witnessed many important changes over the years. With the advent of effective antimycobacterial chemotherapeutic agents in the early 1950s, the two main biological obstacles to successful treatment of TB were the high rate of failure during treatment and the high risk of relapse after treatment. With the introduction of Rifampicin the outlook for treatment of TB changed dramatically. Today's standard regimen directly observed therapy short course (DOTS) according to the WHO/IUATLD consists of a combination of three or four drugs (Isoniazid, Rifampicin, Ethambutol, Pyrazinamide) for two months followed by a dual combination of Isoniazid and Rifampicin for four months[9]

Treatment Categories and Drug Regimens

Standardized Treatment Regimens are one of the pillars of the DOTS strategy[14]

Isoniazid, Rifampicin, Pyrazinamide, Ethambutol, and Streptomycin are the primary antitubercular drugs used. Most DOTS regimens have thrice-weekly schedules and typically last for 6 to 8 months, with an initial Intensive phase and a Continuation phase.

Based on the Nature/severity of the disease and the Patients' exposure to previous anti-tubercular treatments, RNTCP classifies tuberculosis patients in to three treatment Categories.

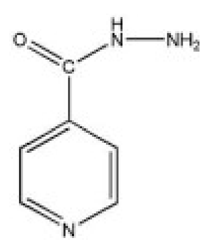
Category I	**Category II**	**Category III**
• New sputum smear-positive • Seriously ill sputum smear-negative • Seriously ill extra-pulmonary • New Sputum Positive/Negative HIV Positive	• Sputum smear-positive Relapse • Sputum smear-positive Failure • Sputum smear-positive Treatment after default • Sputum smear negative Others/Chronic	• New sputum smear-negative, not seriously ill • New extra-pulmonary, not seriously ill
$2H_3R_3Z_3E_3 + 4H_3R_3$	$2H_3R_3Z_3E_3S_3 + 1H_3R_3Z_3E_3 + 5H_3R_3E_3$	$2H_3R_3Z_3 + 4H_3R_3$
2 months Intensive phase + 4 months continuation phase Four drugs at Thrice-weekly Schedule	3 months Intensive phase + 5 months continuation phase Five drugs at Thrice-weekly Schedule	2 months Intensive phase + 4 months continuation phase Two drugs at Thrice-weekly Schedule

H: IsoniaziD (600 mg), R: Rifampicin (450 mg), Z: Pyrazinamide (1500 mg), E: Ethambutol (1200 mg), S: Streptomycin (750 mg)

Second line Drugs[15]

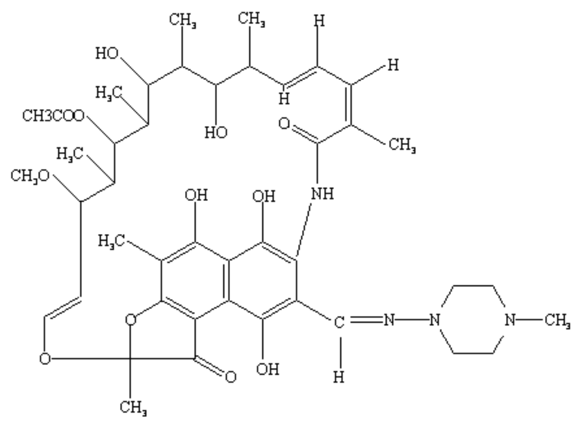
Bactericidal	Bacteriostatic
Amikacin (Am)	Cycloserine (Cs)
Capreomycin (Cm)	p-aminosalycilic acid (PAS)
Ciprofloxacin (Cx)	
Ethionamide (Et)	
Kanamycin (Km)	
Ofloxacin (O)	
Protionamide	

<u>Isoniazid</u>[16] (isonicotinic acid hydrazide; C8H7N3O, MW: 137.1)



Isoniazid is one of the most powerful drugs against TB. INH is a pro-drug that requires processing by the bacterial catalase-peroxidase to become active. Once activated, it inhibits the biosynthesis of mycolic acids, which are essential components of the mycobacterial cell wall. This drug is bactericidal against metabolically active bacilli and bacteriostatic against resting bacilli. INH is active against *M. tuberculosis, M. bovis* and *M. kansasii*. Susceptible *M. tuberculosis* strains show minimal inhibitory concentrations (MIC) between 0.02 and 0.2 mg/ml. Mutations leading to INH resistance have been identified in different gene targets including KatG, inha, ahpc and other genes remain to be established[17]

<u>Rifampicin</u>[16]



RIF, a lipophilic ansamycin, is 5,6,9,17,21-Hexahydroxy-23-mothoxy-2,4,12,16,18,30,22-heptamethyl-8-(N-(4-methyl-1-piperazinylformimidoyl)-2,7(epoxypentadecal(1,11,13) trienimino) naptha (2,1-b) furan-1,11 (2H)-dione-21 acetate, chemically. It is a highly active mycobactericidal drug because it diffuses rapidly across the hydrophobic cell envelope.

RIF inhibits gene transcription, by interacting with the beta subunit of the ribonucleic acid (RNA) polymerase enzyme. It is bactericidal against dividing mycobacteria and also has some activity against non-dividing bacilli. *M. tuberculosis* strains are normally susceptible to 0.1-2 mg/L. The introduction of RIF, thus, allowed reduction of the duration of standard antituberculosis treatments from one year to nine months.

The conformational relationship between the aliphatic chain and the aromatic nucleus is very important for microbiological activity, probably because of the interaction of the drug with its target.

Rifampin (RIF) resistance serves as a surrogate marker for the detection of MDR TB, as 90% of Rif^r isolates are also isoniazid resistant. RIF interferes with transcription and elongation of RNA by binding to the DNA-dependent RNA polymerase. It was observed that resistance to RIF follows a single-step, high-level resistance pattern in which mutations occur spontaneously at a frequency of 1029. The genetic basis for RIF resistance in approximately 95% of the cases is due to mutations in an 81-bp RIF resistance-determining region (RRDR) of the rpoB gene, corresponding to codons 507 to 533 (Escherichia coli numbering system), which codes for the beta subunit of the RNA polymerase of *M. tuberculosis*[18].

DRUG RESISTANCE

Historical aspects of Drug Resistance in *M.tuberculosis*.

Drug resistance as a limiting factor for success of chemotherapy of tuberculosis was recognized almost immediately following monotherapy of Streptomycin, the first effective antituberculous drug[19]. Though there a striking improvement in the patient's symptoms initially, the condition of the patient soon deteriorated with increase in number of bacilli in the sputum. The bacilli isolated at that time instead of being killed by the drug, continued to grow in-vitro, in the presence of high concentrations of the drug.

The classical observation was followed by another important finding by[20], who showed that during treatment with Streptomycin alone, the proportion of drug resistant bacilli increased progressively from about one in 88,750 organisms before the therapy to about one in 367 after 15 weeks of treatment. These observations were the pioneering studies on drug resistance in *M.tuberculosis*.

Subsequent studies by two pioneering scientists[21] showed that with monotherapy or inadequate therapy, the number of susceptible bacilli decreased while the resistant bacilli increased in lung cavities of the patients. This was called the fall and rise phenomenon.

The international group of specialists assembled by the WHO in 1969 adopted the definition of drug resistance of *M.tuberculosis* after testing a large number of wild strains against three drugs available during that period[22]

DEFINITION

Drug resistant tuberculosis is defined as a case of tuberculosis excreting bacilli resistant to one or more anti-tubercular drugs.

Multi-drug resistant tuberculosis (MDR-TB)[5] is defined as disease due to *M.tuberculosis* that is resistant to Isoniazid (H) and Rifampicin (R) with or without resistance to other drugs

Extensively drug resistant tuberculosis (XDR-TB)[23] is resistance to at least Isoniazid and Rifampicin (i.e. multidrug-resistant TB or MDR-TB), plus resistance to any fluroquinolones, and any one of the second-line anti-TB injectable drugs (Amikacin, Kanamycin or Capreomycin).

TYPES OF DRUG RESISTANCE

On epidemiological grounds, drug resistance has been divided into 3 broad categories discussed below. The definitions are used in mass surveys to assess the prevalence of drug resistance in the community or the country. Such surveys conducted at frequent intervals could be used to monitor the success of treatment programs, particularly if a national treatment policy is implemented and thus serve as epidemiological yard sticks[24]. The categories are as follows:

1. Primary Drug Resistance, where the drug-resistant bacilli are isolated from previously untreated patients.
2. Acquired drug resistance, where resistant bacilli are isolated from patients who originally homed in the susceptible bacilli
3. Initial Drug Resistance denotes drug resistance in patients, who deny a history of previous chemotherapy. In actual terms it consists of true primary resistance with a mixture of an unknown amount of undisclosed acquired drug resistance. Because it takes elaborate efforts on the part of the health authorities to obtain information on the possibility of previous treatment, which is unreported, initial drug resistance has been gaining practical importance.

Factors responsible for the development of drug resistance:[25]

The emergence of drug resistance in *M.tuberculosis* has been associated with a variety of management, health provider and patient-related factors.

(i) Deficient or deteriorating TB control programmes resulting in inadequate administration of effective treatment;

(ii) Poor case holding, administration of sub-standard drugs, inadequate or irregular drug supply and lack of supervision;

(iii) Ignorance of health care workers in epidemiology, treatment and control;

(iv) Improper prescription of regimens;

(v) Interruption of chemotherapy due to side effects;

(vi) Non-adherence of patients to the prescribed drug therapy;

(vii) Availability of anti-TB drugs across the counter, without prescription;

(viii) Massive bacillary load;

(ix) Illiteracy and low socio-economic status of the patients;

(x) The epidemic of HIV infection;

(xi) Laboratory delays in identification and susceptibility testing of *M. tuberculosis* isolates;

(xii) Use of nonstandardized laboratory techniques, poor quality drug powders and lack of quality control measures; and

(xiii) Use of anti-TB drugs for indications other than tuberculosis.

Various Gene Loci conferring drug resistance to MTB[26]

Drug	Gene	Gene product/functional role	Cellular target
Rifampicin	rpoB	B-subunit of RNA polymerase/transcription	Nucleic acids
Isoniazid	katG oxyR-ahpC kasA	Catalase-peroxidase/activation of Pro-drug Alkyl-hydro-reductase/unknown b-ketoacyl acyl carrier protein	Cell wall
INH-Ethionamide	inhA	Enoyl-ACP reductase/synthase Mycolic acid synthesis	Cell wall
Streptomycin	rpsL rrs	Ribosomal protein S12 /translation 16S rRNA/translation	Protein synthesis
Fluoroquinolone	gyrA	DNA gyrasde	Nucleic acid
Pyrazinamide	pncA	Amidase/activation of pro-drug	Unknown
Ethambutol	embB	Arabinosyl transferase/arabinan polymerization	Cell wall

LAB DIAGNOSIS

The key to the diagnosis of tuberculosis is a high index of suspicion. Often, the diagnosis is first entertained when the chest radiograph of a patient being evaluated for respiratory symptoms is abnormal. The longer the delay between the onset of symptoms and the diagnosis, the more likely is the finding of cavitary disease. In contrast, immunosuppressed patients, including those with HIV infection, may have "atypical" findings on chest radiography e.g., lower-zone infiltrates without cavity formation.

PPD Skin Testing

Skin testing with PPD is most widely used in screening for *M. tuberculosis* infection. The test is of limited value in the diagnosis of active tuberculosis because of its low sensitivity and specificity. False-negative reactions are common in immunosuppressed patients and in those with overwhelming tuberculosis. Positive reactions are obtained when patients have been infected with *M. tuberculosis* but do not have active disease and when persons have been sensitized by nontuberculous mycobacteria or bacille Calmette- Gue´rin (BCG) vaccination[27]

A presumptive diagnosis is commonly based on the finding of AFB on microscopic examination of a diagnostic specimen such as a smear of expectorated sputum or of tissue

Diagnosis of Latent Tuberculosis Infection

QuantiFERON®-TB gold (The Whole Blood IFN-gamma Test Measuring Responses to ESAT-6 & CFP-10 Peptide Antigens)

QuantiFERON®-TB Gold is an indirect test for *M. tuberculosis* infection (including disease) and is intended for use in conjunction with risk assessment, radiography and other medical and diagnostic evaluations.

The QuantiFERON®-TB Gold is a test for Cell Mediated Immune (CMI) responses to peptide antigen cocktail simulating the mycobacterial proteins ESAT-6 and CFP-10. These proteins are absent from all BCG strains and from most non-tuberculosis mycobacteria with the exception of *M. kansasii, M. szulgai* and *M. marinum*. Individuals infected with *M. tuberculosis* complex organisms usually have lymphocytes that recognise these mycobacterial antigens. This recognition process involves the generation and secretion of the cytokine, IFN-γ. The detection and subsequent quantification of IFN forms the basis of this test.

The QuantiFERON®-TB Gold test is both a test for LTBI and a helpful aid for diagnosing *M. tuberculosis* complex infection in sick patients. A positive result supports the diagnosis of tuberculosis disease; however, infections by other mycobacteria (e.g., *M. kansasii*) could also lead to false positive results[28].

<u>SAMPLE COLLECTION</u>[29]

The number of specimen required for diagnosis of smear positive pulmonary TB is two, with one of them being a morning sputum specimen.

Two sputum specimens are collected over one, or two consecutive days. Of the two sputum specimens, one is collected on the spot and the other is an early morning specimen collected at home by the patient. One specimen positive out of the two is enough to declare a patient as smear positive TB. Smear positive TB is further classified as a new or re treatment case based on their previous treatment history, and appropriate therapy is prescribed.

Other respiratory specimens include induced sputum, tracheal aspirate, bronchoalveolar lavage, pleural fluid and lung biopsy tissue.

AFB microscopy

Most modern laboratories processing large numbers of diagnostic specimens use auramine-rhodamine staining and fluorescence microscopy. The more traditional method -light microscopy of specimens stained with Kinyoun or Ziehl-Neelsen basic fuchsin dyes- is satisfactory, although more time-consuming.

Grading of AFB smears by Z-N microscopy[30]

No of acid-fast bacilli (AFB)	Fields	Report
No AFB	In 100 immersion fields	Negative
1-9 AFB	In 100 immersion fields*	Record exact figure (1-9 AFB/ 100 fields)
10 to 99 AFB	In 100 immersion fields	1+
1 to 10 AFB	Per field (examine 50 fields)	2+
More than 10 AFB	Per field (examine 20 fields)	3+

DETECTION OF MTB

Processing of sample - concentration methods

`The most commonly used decontamination and concentration method for sputum samples are Modified Petroff's method and NALC (N Acetyl L cysteine) method.

Conventional culture method

For detection of Mycobacteria in clinical specimens the current "gold standard" consists of a combination of solid and liquid media[9]. In detecting as few as $10^1 – 10^2$ viable organisms per ml of specimen in the optimal case, culture is more sensitive than smear. Also it is the only reliable means to monitor effectiveness of therapy in TB patients[31]

Specimens may be inoculated onto egg- or agar-based medium (e.g., Lowenstein-Jensen or Middlebrook 7H10) and incubated at 37ºC under 5% CO2. Because most species of mycobacteria, including *M. tuberculosis*, grow slowly, 4 to 8 weeks may be required before growth is detected. Although *M. tuberculosis* may be presumptively identified on the basis of growth time and colony pigmentation and morphology, a variety of biochemical tests have traditionally been used to speciate mycobacterial isolates. In today's laboratories, the use of liquid media for isolation and speciation by nucleic acid probes or high-pressure liquid chromatography of mycolic acids (HPLC) has replaced the traditional methods of isolation on solid media and identification by biochemical tests. These new methods have decreased the time required for bacteriologic confirmation to 2 to 3 weeks[13]

Rapid and sensitive detection of *Mycobacterium tuberculosis* is of clinical importance for the treatment, control, and prevention of tuberculosis. Despite new nucleic acid amplification assays, unequivocal diagnosis of tuberculosis continues to rely on cultivation of *M. tuberculosis*.

Automated culture method[32]

The fully automated systems that allows continuous monitoring of mycobacterial cultures are

1) BACTEC 460

2) MB/BacT

3) ESP culture system II

The **BACTEC 460 system** (Becton Dickinson, Sparks, Md.) has been marketed since 1977. The Middle brook 12B medium for this system contains ^{14}C labeled palmitic acid as the substrate. During mycobacterial growth, ^{14}C -labeled $^{14}CO_2$ is produced and released into the headspace air of the vials. A $^{14}CO_2$ detection device allows early determination of mycobacterial growth. However, this system requires radioactive reagents, causing waste problems, and vials have to be handled and punctured for readings at least eight times during 6 weeks of incubation, requiring a considerable amount of work and increasing the risk of cross contamination.

The recently developed **MB/BacT system** (Organon Teknika, Turnhout, Belgium) relies on a continuous colorimetric CO_2detection device to indicate mycobacterial growth in a closed system. A solid-state sensor at the base of each vial contains the colorimetric indicator, which changes from green to yellow when CO_2 is produced in the vial. Each compartment of the instrument where the vials are incubated contains a reflectometer and a detection unit.

ESP culture system II is based on the detection of pressure changes in the head space above the broth medium resulting from gas production or consumption due to growth of micro organisms

Other phenotypic detection methods

The fast plaque TB (Biotech Labs Ltd., Ipswich, UK) is a rapid manual test for the detection of *M. tuberculosis* from clinical specimens within 48 h[33]. This test utilizes specific mycobacteriophages (Actiphage™) to reflect the presence of viable *M. tuberculosis*. Mycobacteriophages are added to a clinical specimen and allowed to incubate for one hour to allow phage infection of target tubercle bacilli. After the incubation period, a virucidal solution (Virusol™) is added, which destroys all phages that have not infected the bacilli. The remaining phages replicate in the infected bacilli until new progeny phages are released as the cells lyse. The progeny phages are amplified by the addition of a non-pathogenic rapidly growing mycobacterial host *M. smegmatis* (Sensor™ cell), which is also able to support phage replication. This is visualized as plaques, which are clear areas in a lawn of Sensor™ cell growth. The number of plaques visualized is directly related to the number of viable tubercle bacilli in the original sample.

Mycolic acid analysis (HPLC)

Mycolic acids are present in all mycobacteria. Their composition is constant for all strains of a given species and varies from species to species. An HPLC method for analysis of mycolic acid esters, has been standardized and demonstrated to be a rapid and reliable method for identification of many mycobacterium species[34]

In this procedure, mycolic acids are extracted from saponified mycobacteria, converted to p-bromophenacyl esters, and analysed by HPLC. The mycolic acid esters are separated on a reversed-phase C18 column by a methanol-methylene chloride gradient elution and detected by UV or fluorescence detection spectrophotometry.

The standardized method recommends a visual comparison of a sample HPLC pattern on an atlas of reference strain patterns in combination with the use of peak height ratios.

GENOTYPING METHODS FOR DETECTION OF M.TUBERCULOSIS [35]

DNA probes:

Based on information about specific gene sequences well defined oligonucleotide probes for identification of various clinically relevant mycobacteria have been developed and are readily available. These probes are being used in several countries for rapid confirmation of the identity of mycobacterial isolates. When used along with newer methods of detection of the early growth (such as BACTEC, Septi-Chek, MGIT) these are of great help in rapidly confirming the diagnosis as identity of isolate can be established within 1 to 2 days with gene probes as compared to much longer time required with classical biochemical tests

INNO LIPA

The LiPA assay is based on the principle of reverse hybridization. Biotinylated DNA material, obtained by means of a PCR amplification of the 16S-23S ribosomal RNA spacer region, is hybridized with 14 specific oligonucleotide probes immobilized as parallel lines on membrane strips. The addition of streptavidin labeled with alkaline phosphatase and of a chromogenic substrate results in a purple-brown precipitate on hybridized lines**.**

Gene amplification methods for direct detection of *M.tuberculosis* sequences from clinical specimens:

Gene amplification techniques have made a major impact on the diagnosis of mycobacterial diseases. These methods may be classified as those based on polymerase chain reaction (PCR) and others based on isothermal amplification reactions. Gene amplification techniques are highly sensitive and under optimum conditions may detect 1-10 organisms. If systems are adequately standardized, evaluated and precautions for avoiding the contamination are taken, these assays can play a very useful role in early confirmation of diagnosis in paucibacillary extra-pulmonary forms of tuberculosis.

PCR methods

A variety of PCR methods have been developed for detection of specific sequences of *M.tuberculosis* targeting either DNA or rRNA/and these could be based on conventional DNA based PCR, nested PCR and RT-PCR. Targets include insertion and repetitive elements, various protein encoding genes, ribosomal rRNA etc. A large number of PCR assays targeting different gene stretches of *M. tuberculosis* have been described. Different

Indian investigators have used separate gene targets like MPB 6433, repetitive sequences , GC repeats , devR , 38kD , TRC [4] , IS 1081 and a system patented by Central Drug Research Institute (CDRI), Lucknow. Some of these assays (CDRI) have been repeatedly found to be reproducible, highly sensitive and specific in double blind evaluations. IS-1081 based system has been further modified and a new nested PCR target of this gene has been developed at Centre for DNA Fingerprinting and Diagnostics (CDFD), Hyderabad. This strategy can be used for confirming the diagnosis and also monitoring the progress.

Isothermal amplification techniques:

These techniques represent a major step forward in the application of gene amplification technology without thermal cycler. The important methods are:

(i**) Strand displacement amplification (SDA):**

It is an isothermal in vitro nucleic acid amplification technique which is based upon the ability of HincII to nick the unmodified strand of hemiphosphorothiote form of its recognition site and the ability of exonuclease deficient klenow to extend 3’ end at the nick and displace the downstream DNA strand. Exponential amplification results from coupling sense and antisense reactions.

(ii**) Gene probe amplified *Mycobacterium tuberculosis* direct test:**

This assay employs the isothermal amplification of *M. tuberculosis* complex rRNA followed by detection of amplicon with acridinium ester-labelled DNA probe. Reports about the application of this system are encouraging.

(iii) **Q-beta (QB) replicase based gene amplification**:

This approach involves production of RNA in the amplification reaction using QB replicase as the enzyme and reaction at fixed temperature (for example 37°C). Using a suitable combination of capture and detector probes, sensitivity up to one colony forming unit has been reported for *M. tuberculosis*. Further, the inhibitors of PCR were observed to have no effect on this assay.

Gene amplification methods for identification and speciation of *M.tuberculosis*

Different strategies to identify the isolates from cultures and directly from the clinical specimens have been described. These include amplification of specific gene regions followed by hybridization with species specific probes, sequencing and RFLP analysis such as hsp 65 kDa gene 21, katG22 and rRNA genes (23-25, a CJIL system under publication) have been described. These PCR-RFLP assays help in quick identification of pathogenic mycobacteria including *M. tuberculosis* from the culture isolates as well as directly from the clinical specimens. While PCR-sequencing approach can be applied by reference laboratories the hybridization and RFLP approaches are easily practicable in clinical mycobacteriology laboratories.

DRUG SUSCEPTIBILITY TESTING METHODS

Phenotypic methods[26]

Absolute concentration method:

This method uses a standardized inoculum grown on drug free media and the media containing graded concentrations of the drug to be tested. Several concentrations of each drug are tested, and the resistance is expressed in terms of the lowest concentration of the drug that inhibits the growth; i.e., minimum inhibitory concentration (MIC). This method is greatly affected by the viability of organisms.

Resistance Ratio method:

It compares the growth of unknown strains of *M.tuberculosis* with that of standard laboratory strain (H37Rv). Parallel sets of media containing two-fold dilutions of the drug are inoculated with the standard strains of tubercle bacilli. Resistance is expressed as the ratio of the MIC of the test strain to the MIC of the standard strain in the same set. This test is also greatly affected by the inoculum size as well as the viability of the strains. In addition any variation in the susceptibility of the standard strain also affects the RR of the test strain.

Proportion method:

This method enables a precise estimation of the proportion of mutants resistant to a given drug. Several 10-fold dilutions of the inoculum are planted on to both control (drug free) and the drug containing media. At least one dilution should yield isolated countable (50-100) colonies. When these numbers are adjusted by multiplying the dilution of the inoculum used, the total number of viable colonies on the control medium and the number of mutant colonies resistant to the drug concentrations tested may be estimated. The proportion of the bacilli resistant to a given drug is then estimated by expressing the resistant portion as a percentage of the population used.

Microscopic Observation Drug Susceptibility Assay (MODS)[36]:

The microscopic-observation drug-susceptibility (MODS) assay is a low-cost, low-tech tool for high-performance detection of TB and MDRTB. The MODS assay is based on three principles: 1) *M.tuberculosis* grows faster in liquid media 2) microscopic *M.tuberculosis* growth is characteristic and can be detected earlier in liquid 3) the drugs Isoniazid and Rifampicin can be incorporated into the MODS assay to allow for simultaneous direct detection of MDRTB, obviating the need for subculture to perform an indirect drug susceptibility test.

Pha B assay:

This is a new phenotype culture drug susceptibility testing method named as phage amplified biologically (Pha B), and is based on the ability of viable *M.tuberculosis* to support the replication of an infecting mycobacteriophage:noninfecting exogenous phages are inactivated by chemical treatment. The number of endogenous phages, which is an indication of the original number of viable *M.tuberculosis*, is determined after cycles of infection, replication and release in rapidly growing mycobacteria. In the case of drug resistant *M.tuberculosis*, bacilli will remain viable and protect the mycobacteriophage. Any mycobacteriophage protected within viable bacilli replicate and ultimately lyse their host. For rapid detection, the released mycobacteriophages are mixed with rapidly growing *M.smegmatis* host in which they undergo rapid cycle of infection, replication and lysis. Lysis is easily seen as clear areas or plaques in a lawn culture of *M.smegmatis*. The number of plaques generated from a given sample is directly proportional to the number of protected mycobacteriophages, which is dependent on the number of tubercle bacilli that remain viable after drug treatment.

Luciferase reporter phage assay:

In this technique, viable mycobacteria are infected with reporter phages expressing firefly luciferase gene. Easily detectable signals are seen a few minutes after the infection of *M.tuberculosis* with reporter phages. Light production requires metabolically active *M.tuberculosis* cells, in which reporter phages replicate and luciferase gene is expressed. When drug susceptible *M.tuberculosis* strains are incubated with specific anti-tuberculosis drugs, they fail to produce light after infection with luciferase reporter phages. In contrast, drug resistant strains are unaffected by the drugs and produce light at the levels equivalent to those documented for untreated controls after infection with reporter phages. The other reporter molecules described is the green fluorescence protein (GPF) of the jellyfish Aequorea Victoria. This reporter system does not require cofactors or substances due to intrinsic fluorescence nature of the GPF. Luciferase reporter tests have now been evaluated against the four first-line antibiotics with an overall agreement of 98.6% compared with the BACTEC TB_460.

Colorimetric methods:

Colorimetric methods are based on the reduction of a colored redox indicator added to the culture medium after MTB has been exposed invitro to different drugs. Resistance is detected by a change in colour of the indicator, which is directly proportional to the number of viable mycobacteria in the medium

Different indicators have been evaluated for testing against first and second-line drugs, giving comparable results in agreement with the gold standard proportion method. In a multicentre evaluation to assess two colorimetric methods using the **MTT** (3-(4,5-dimethylthiazol-2-yl)2,5-diphenyltetrazolium bromide) and **resazurin** as redox indicators for the first line drugs, very good results were obtained with sensitivity and specificity of 99 and 96% respectively[37].

The Nitrate Reductase assay

The nitrate reductase assay (NRA) is a very simple technique based on the capacity of *M.tuberculosis* to reduce nitrite. *M.tuberculosis* is cultivated in the presence of an antibiotic and its ability to reduce nitrate is measured after 10 days of incubation. Resistant strains will reduce the nitrate, revealed by a pink-red colour in the medium, while susceptible strains will lose this capacity as they are inhibited by the antibiotic[38].

The test was recently evaluated for first and second line drugs with good results. The test performed very well for Isoniazid, Rifampicin and Ethambutol with accuracy between 96.6% and 98%. The main advantage of the NRA, in addition to its simplicity, is that it uses the same format and culture medium as used in the conventional method, facilitating its implementation in diagnostic laboratories.

The MGIT system, in its manual or automated version and based on the measurement of oxygen consumption, has been thoroughly evaluated for DST of *M.tuberculosis* to first and second line drugs showing a good concordance with the gold standard PST method[39]

Radiometric method to detect early growth of mycobacteria in culture is the BACTEC 460 system, wherein C_{14} labelled palmitic acid in 7H12 medium is used. The system detects the presence of mycobacteria based on metabolism of C_{14} rather than visible growth. This method provides rapid growth, specific identification and drug susceptibility testing within 14 days[26]. The most common reported problems associated with BACTEC 460 include: the risk of needle puncture, the need to dispose radioactive waste, and potential contamination of test samples

Molecular Assays for detection of drug resistance.

DNA sequencing:

DNA sequencing of PCR-amplified products has been the most widely used method, becoming the gold standard. It has been performed by both manual and automated procedures although the latter has been the most commonly used. It has been thoroughly used for characterising mutations in the rpoB gene in Rifampicin resistant strains and to detect mutations causing resistance other anti-TB drugs. Several other genotypic methods, like PCR-single strand conformation polymorphism, PCR-heteroduplex formation and solid phase hybridisation assays have been developed.

Moreover, the **line probe assay(LiPA-RIF)** based on the hybridisation of amplified DNA from cultured strains or clinical samples to ten probes encompassing the core region of the rpoB gene of *M.tuberculosis*, immobilised on a nitrocellulose strip and the Genotype mycobacterium tuberculosis test (MTB) DR (Hain, Germany), a commercial system for the detection of the MTBC and its resistance to Rifampicin and INH from culture samples based on the detection of the most common mutations in the rpoB gene and katG genes, respectively.

Genotype MTBDR Strip based test:

The genotype MTBDR (Hain Life Science, Nehren, Germany) is a new commercial and easy to perform assay developed for the detection of RIF and/or INH resistance in TB strains. The test is based on reverse hybridisation between amplicons derived from a multiplex PCR and nitrocellulose bound wild-type and mutated probes for the mutations of interest. The test allows rapid and specific detection of most mutations conferring resistance not only to RIF but also to INH in *M. tuberculosis* isolates. This test combines the probes targeting the 81-bp "hot-spot" region in the rpoB gene, with two probes targeting two different mutations at position 315 of the katG gene. Mutations affecting this codon are responsible for INH resistance in ca.60% of the cases worldwide. The simultaneous detection of resistance to both RIF and INH allows an early diagnosis of an MDR case[40]

More recently DNA microarrays or high-density oligonucleotide arrays have been applied for species identification of mycobacteria. The method is based on hybridisation of fluorescently-labelled PCR amplified products obtained from mycobacterial colonies to DNA arrays containing nucleotide probes

Real-time Polymerase Chain Reaction Techniques:

Real time PCR techniques have also been introduced for rapid detection of drug resistance. Different probes have been used like the TaqMan probe, fluorescence resonance energy transfer (FRET) probes, molecular beacons and bio probes. The main advantages of real time PCR techniques are the speed of the test and a lower risk of contamination. The main disadvantages would be requirement for expensive equipment and reagents, and the need for skilled technical personnel. In several studies, real time PCR has been evaluated in culture material and more recently in clinical samples. The sensitivity in these studies has ranged from 71-98% with specificity close to 100 %[41]. The main advantage of the RT-PCR is its speed in giving results, 1.5-2 h after DNA extraction, and the decrease in the risk of contamination since both reaction and detection occurs in a single tube. Further studies are necessary to confirm the real value of this new methodology in the clinical setting.

Miscellaneous genotyping method:

These include new genotypic techniques for the rapid detection of drug resistance in *M.tuberculosis*. Cleavage fragment length polymorphism (CFLP), dideoxy finger printing (ddF), hybridization protection assays, a technique based on reverse transcriptase strand displacement amplification of m-RNA, RNA-RNA duplex base-pair mismatch assay and DNA sequence analysis using flurogenic reporter molecules. However, these techniques have not been extensively studied and have not been further validated with clinical isolates. Although they share a high specificity common to all sequencing techniques, most of them rely on technically demanding procedures and in some cases need specialized and costly equipment precluding their use in laboratories.

Materials & Methods

MATERIALS AND METHODS[42]

A total of 117 respiratory specimens from patients attending the RNTCP outpatient clinic and from inpatients at PSG Hospitals during the period between June 2008 and June 2009 were included in the study. Human ethical clearance for the study was obtained from the Institutional ethical committee prior to collection of samples. A copy of the certificate is attached. The respiratory samples included were sputum, bronchoalveoaler lavage and pleural fluid.

Sample collection

About 5 to 10 ml of well coughed up early morning and spot sputum samples without salivary contamination was collected in a sterile wide mouthed, screw-capped container. BAL and Pleural fluid were received in a sterile container. In case of a delay in processing immediately the samples were stored at 4ºC for not more than 24 hours.

All specimens that were positive for acid fast bacilli by Ziehl Neelsen staining of smears were included in the study. Processing of all samples was done in a bio safety cabinet class IIB with Universal precautions.

Ziehl-neelsen staining procedure

A smear was made on a clean slide from thick purulent part of the sample. The smears were dried, heat fixed and stained by the Ziehl-Neelsen technique.

1. Slides were placed on a staining rack with the smeared side facing up, ensuring that the slides were not touching each other
2. Slides were flooded with strong carbol-fuchsin, which was filtered before use
3. Slides were heated intermittently until steaming for 5-7 minutes. Care was taken to see that the stain did not boil or dry out.
4. Each slide was rinsed individually in a gentle stream of distilled water until all free stain is washed away
5. Slides were flooded with the decolorizing solution (25 % sulphuric acid) for 2-3 minutes.
6. Slides were rinsed again thoroughly with water. Excess water was drained off.
7. Slide was flooded with methylene blue counter stain for 30 seconds.
8. The slides were air dried after rinsing thoroughly and draining off excess water.
9. Under the oil immersion objective, acid fast bacilli were seen as slender pink rods while the non acid fast bacilli and the background appeared blue in colour.

Smear examination

Samples were examined by direct microscopy after staining by Ziehl Neelsen Stain and examined under oil immersion. Sputum smears were graded depending on the number of bacilli seen

Table 1: Grading of AFB smears by Z-N microscopy

No of acid-fast bacilli (AFB)	Fields	Report
No AFB	In 100 immersion fields	Negative
1-9 AFB	In 100 immersion fields*	Record exact figure (1-9 AFB/ 100 fields)

10 to 99 AFB	In 100 immersion fields	1+
1 to 10 AFB	Per field (examine 50 fields)	2+
More than 10 AFB	Per field (examine 20 fields)	3+

Presence of acid fast bacilli >1 was taken as positive for BAL and Pleural fluid.

All sputum and BAL samples were decontaminated by Modified petroff's method and 1-2 loopful of the deposit was inoculated on two Lowenstein Jensen medium using 4mm diameter nichrome loop. Pleural fluid specimens were centrifuged at 3000g for 15 minutes and the sediment was used as inoculum.

Sodium hydroxide (modified petroff's) method:

- To *x* ml of sputum, *2x* ml of 4% NaOH was added and mixed well
- The tubes were kept for 15 minutes at 37ºC with occasional shaking
- The tubes were centrifuged at 3000 g for 15 minutes and the supernatant was poured off taking care not to discard the sediment.
- Approximately 20 ml sterile distilled water was added to resuspend the sediment and the tubes were centrifuged again at 3000 g for 10 minutes
- Supernatant was decanted and deposit was inoculated onto two slopes of L-J medium.

Culture

- The bottles were kept in racks and incubated for 8 weeks at 35-37ºC under aerobic atmosphere.

- It was then examined daily for 1 week (for any rapid growers, or for contamination, indicated by bluing, digestion of media, etc). Hand lens was used.
- The colonies of *Mycobacterium tuberculosis* were dry, rough, irregular colonies with a wrinkled surface and buff colour.

REPORTING OF POSITIVE, NEGATIVE AND CONTAMINATION RESULTS

Reading	Report
No growth	Negative
1-19 colonies	Positive (number of colonies)
20-100 colonies	Positive (1+)
>100 discrete colonies	Positive (2+)
Confluent growth	Positive (3+)
Contaminated	Contaminated

A total of 104 positive cultures resembling *M.tuberculosis* were subjected to biochemical tests and susceptibility to p-nitro benzoic acid for its identification

Biochemical tests

The following biochemical tests were used in identification of *Mycobacterium tuberculosis* complex.

NIACIN PRODUCTION TEST

A culture must be at least three to four weeks old and must have sufficient growth of at least 100 colonies.

Procedure

1. All the slopes were arranged and 1 ml sterile distilled water was added to the tube and dug to extract niacin
2. The bottles were left in slanting position for 30 minutes
3. The bottles were kept upright for 10min to allow the fluid to drain to bottom
4. 0.5 ml of the culture extract was pipetted into a clean screw-capped tube placed on a rack inside the Bio Safety Cabinet.
5. 0.5 ml of 4% aniline in alcohol and 10% cyanogen bromide were added sequentially
6. The contents were mixed well and checked for appearance of canary yellow colour.

Controls:

Positive control: Extract from culture of *M.tuberculosis* $H_{37}Rv$ strain

Negative control: Extract from uninoculated bottle of medium.

CATALASE TEST at 68ºC/pH 7.0

Procedure

1. With a sterile pipette, 0.5 ml of 0.067 M buffer was added aseptically into 16 x 125 mm screw-capped test tubes
2. A loopful of the test culture was suspended in the buffer solution, using a sterile loop

3. The tubes with the emulsified culture were placed in a previously heated water bath at 68ºC for 20 minutes.

4. The tubes were removed from water bath and cooled to room temperature

5. Catalase reagent (0.5ml) was added and the tubes were observed for the formation of bubble appearing on the surface of the liquid. Tubes were not shaken because Tween-80 may also form bubbles when shaken, resulting in false positives.

6. Negative tubes were held for 20 minutes before discarding

Control:

Positive control: strain *M.terrae* complex

Negative control: strain *M.tuberculosis*, $H_{37}Rv$ strain

NITRATE REDUCTION TEST

Procedure

One loopful of culture was emulsified in one test tube containing 2 ml of the sterile substrate (sodium nitrate)

The contents were mixed well and incubated upright in a water bath maintained at 37ºC for 2 hours

After 2 hours, the tubes were brought to room temperature. And the reagents were added in the following order:

1 drop of 1:1 HCl

- 2 drops of 0.2% sulphanilamide solution
- 2 drops 0.1% N- (1-naphthyl)-ethylene diamine di-HCl

Development of a pink to red color indicated a positive reaction.

All negative reactions were confirmed by adding a pinch of zinc dust.

Development of a pink color at this stage indicated that the initial negative reaction was true negative. If no color change occurred after adding the zinc dust, the reaction has proceeded beyond nitrite into other components. The entire test was repeated.

Controls

Positive control : strain *M.tuberculosis*, $H_{37}Rv$ strain

Negative control: reagent control without organisms

SUSCEPTIBILITY TO P-NITROBENZOIC ACID (PNB)

Procedure

- Two slopes of LJ medium without drugs and one slope of LJ medium containing *p*-nitrobenzoic acid (PNB) at a concentration of 500µg/ml were inoculated with the neat bacterial suspension equivalent to 0.5 Mcfarlands standard and incubated at 37^0C.
- Reading was taken after 28 days.

Results and interpretation

M.tuberculosis does not grow on PNB medium.

All other mycobacteria are resistant.

Drug susceptibility test

Drug susceptibility testing to Isoniazid and Rifampicin was done by absolute concentration method. Standardization of procedure was done with standard strain $H_{37}Rv$, known sensitive and multidrug resistant strains (for drug concentration in media, inoculum size, and optimum incubation period required for sensitivity testing of both RIF and INH).

Once standardized, the results were applied to 101 consecutive clinical isolates sensitive to PNB after being identified as *M. tuberculosis* by culture and biochemical methods.

- Test media used: Lowenstein-Jensen medium with drug concentrations as given below.

Table 2: Drug concentrations (µg/ml)

Drugs	$H_{37}Rv$	Test strain
Isoniazid	0.025, 0.05, 0.1, 0.2, 1.0, 5	0.2, 1, 5
Rifampicin	4, 8, 16, 32, 64, 128	32, 64, 128

- Anti mycobacterial drugs tested - Rifampicin (RIF) and Isoniazid (INH) obtained from Sigma laboratories.

Absolute concentration (MIC) method

Bacterial suspension

- Sterile tubes labeled with lab number of the culture to be tested were taken.
- Using sterile pipette 0.2ml of sterile distilled water was added to each tube, each containing 6- 10 glass beads.
- Using a 2 mm internal diameter 24 SWG wire loop, a loopful of representative sample of the bacterial mass which is approximately equal to 4 mg moist weight was gently dispersed into the tube.

- The tube was vortexed for 30 to 60 seconds to produce a uniform suspension.
- Gently shake the tube after adding 0.8 ml of sterile distilled water. The suspension was left inside the cabinet for 15-20 minutes for the coarser particles to settle down.
- Using a 4mm external diameter 27 SWG nichrome wire loop, one loopful of this suspension was inoculated on each slope of the test media containing graded concentrations of the drug.
- Two drug free slopes (control) and one drug containing slope of each concentration of the drug(s) was inoculated for each strain tested.

•...The standard strain H37Rv was tested with each batch of test.

- The lab number of the cultures was recorded in the DST register with date.

Incubation and reading of the tests

•...The inoculated slopes were incubated and looked for growth after 28 days of incubation.

•... In this method 'growth' is defined as the presence of 20 colonies or more.

•...As each slope was examined, the results were recorded as follows:

3+	Confluent growth
2+	Innumerable colonies (>100 colonies)
1+	20-100 colonies
1-19	Actual number of colonies

- If the drug-free control slope yielded 100 colonies or less, the test was repeated from the control slope.
- The lowest concentration of the drug inhibiting growth (MIC) was recorded
- The MIC at which the isolates were considered resistant were 0.2µg/ml for Isoniazid and 32µg/ml for Rifampicin

MTT ASSAY[43]

A total of 70 isolates of *Mycobacterium tuberculosis* strains obtained from the sputum samples were tested by MTT method. The test was standardized with respect to the inoculum size and optimum incubation period required for sensitivity testing of both RIF and INH

Four known *Mycobacterium tuberculosis* strains tested by absolute concentration method (Two sensitive i.e., sensitive control-SC and two resistant i.e., resistant control-RC to both the drugs) and one standard sensitive (SS) H_{37} Rv strain received from the stock cultures were used.

- Test media used: Middle brook 7H9 broth supplemented with 10% OADC (Oleic acid, albumin, dextrose and catalase) and 0.01% glycerol (Hi Media).
- MTT solution- (3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) obtained from Hi Media.

Standardization of Inoculum Size

An inoculum of about 8 ml was prepared by suspension of colonies from three to four week- old LJ slopes in 7H9 medium for SC (Sensitive control), RC (Resistant control) and SS (Standard sensitive- H37RV strain). Different concentrations ranging from 10^{7} cfu/ml (McFarland no.1), to 10^{5} cfu/ml were made by serial tenfold dilution. Each concentration was sub cultured on 5% sheep blood agar to rule out contamination by other organisms before putting up the test. Each concentration containing 8 ml suspension was then distributed in seven sterile screw cap tubes in 1ml amount and incubated for 7 days at 37°C.

Every day one of the tubes from each concentration was tested by MTT assay and tubes were inspected for appearance of purple colour. From this experiment, a concentration of 10^{7} cfu/ml was found to give the colour change at the earliest and hence was taken as the standard inoculum size for the further testing.

Standardization of the Incubation Time

To optimize the period of incubation for each drug, the test was put in four sets of four tubes (i.e**.** Resistant control , Sensitive control**,** Drug free control and Blank control) and the tubes were incubated at 37°C for 2, 3, 4, 6 days for RIF and 4, 6, 7, and 9 days for INH, prior to the MTT assay. For RIF testing, 0.5 ml of standardized inoculum was added to 0.5 ml of RIF solution containing a concentration of 2µg/ml so as to get a final concentration of 1µg/ml. To obtain 0.2µg/ml of final drug concentration for INH, 0.1 ml of inoculum was added to the tube containing 0.9 ml solution i.e., 0.4 ml plain 7H9 and 0.5 ml drug medium. For each of the resistant and sensitive isolate tested, drug free controls containing 0.5 ml of standard inoculum and 0.5 ml of plain 7H9 were included. Blank controls containing only drug solution and media were set up for colorimetric reading.

Procedure

The assay was performed by adding 10µl of MTT solution into each tube (i.e., drug containing tubes, drug free control tubes and blank control) on the respective days of reading and the tubes were incubated for four hours at 37°C. One ml of solubilising solution containing 0.1N Hydrochloric acid in isopropanolol was then added to the tubes and the contents were mixed thoroughly by inverting the tubes. After half to one hour of incubation at room temperature, any change in colour to purple in each tube was recorded visually. The strain showing colour change to purple in both control and drug containing tube was labeled as resistant while strain showing colour change in drug free controls and no change in drug containing tube was labeled as sensitive strain.

GENOTYPIC METHODS

1. PCR

Cultures which showed resistance to both drugs and resistance to Rifampicin were subjected to DNA extraction, amplified using specific primers targeting the rpoB gene 81bp region and sequenced for identification of mutation

a. Extraction of DNA from *Mycobacterium tuberculosis*[44]

1. One to two loopful of the colonies from LJ slopes were suspended in 0.5 ml of sterile Milli Q water, in a 1.5 ml Eppendorf tube.
2. The tubes were kept at 85° C for 30 minutes in a waterbath.
3. The tubes were centrifuged at 10000 RPM for 10 minutes at 25° C and the supernatant was discarded. To the pellet, 100 µl of TE buffer was added.

4. The contents were treated with 50µl of lysozyme (20mg/ml) and incubated at 37° C for 1hour to degrade the cell wall.
5. 70µl of 10% SDS + 6µl of 10 mg/ml proteinase K were added to precipitate the proteins.
6. The tubes were vortexed until the pellet was redistributed and later incubated for 10 minutes at 65° C.
7. 100µl of 5M NaCl + 80µl of CTAB-NaCl was added to precipitate polysaccharides and residues and incubated at 65° C for 10 minutes.
8. To the tubes, 800 µl of chloroform ($CHCl_3$) + iso-amyl alcohol (24:1) was added [used to remove proteins from preparations of nucleic acids. $CHCl_3$ denatures proteins, while iso-amyl alcohol reduces foaming during extractions and facilitates separation into aqueous and organic phase].
9. The tubes were centrifuged at 10000 RPM for 10 minutes.
10. The supernatant was transferred to a fresh tube and 600µl of ice cold iso-propanolol was added (to precipitate the DNA).
11. The tubes were incubated at -20° C, overnight.
12. The next day the tubes were centrifuged at 12000 rpm for 10 minutes at 4^0C
13. The contents were discarded and the pellet was washed with 1 ml of 70% ice-cold absolute alcohol (if too much of protein contamination occurred, this step was repeated twice).

14. The contents were discarded, by tapping on a clean filter paper and the tubes were kept open at 45° C until dry.

15. The resulting pellet was re-suspended in 40µl of TE buffer until required for usage.

b. Amplification by PCR

Primer used for amplification of 81bp region on the rpoB gene

Forward primer 5’ GGTGGAAACCGACGACATCGA 3’

Reverse primer 5’ CGCATCGATCGGCGAATTGG 3’

Table 3: Volume of reagents used

PCR master mix	1X	For 9 samples – 10X
Taq Buffer	2µl	20µl
dNTP mix	0.8 µl	8 µl
Primer – forward (200mM)	1 µl	10 µl
Primer – backward (200mM)	1 µl	10 µl
Taq DNA Polymerase	0.4 µl	4 µl
Water	12.8 µl	128 µl
Total	19 µl	

19 µl of the master mix was aliquotted to each labeled PCR tube. 1 µl of DNA was added to the corresponding tubes and PCR amplification was run using Eppendorf thermocycler.

c. PCR cycle

Each cycle comprised of an initial denaturation at 94 º C for 5 minutes and 40 cycles of denaturation at 94 ºC for 45 sec, annealing at 65 ºC for 50sec and amplification at 72 ºC for 45 sec with a final extension at 72 ºC for 5 minutes

Detection of PCR products by gel electrophoresis

At the end of the reactions, a 1.5% gel was prepared with agarose and the PCR products were run using the electrophoresis kit to look for amplified products.

1.5% Gel preparation

1. To 20 ml of 1X TBE buffer, 300mg of agarose was added and the contents were heated in a microwave until it formed a clear solution.
2. To this 1µl of ethidium bromide was added and the solution was poured into a trough with comb.
3. The solution was allowed to set for approximately 30 minutes.
4. Once set, the comb and the tape around the trough was removed.
5. The trough was placed in an electrophoresis tank containing 1X TBE buffer. The trough should just immerse in the buffer.
6. Loading dye was mixed with 500bp ladder and each extracted DNA separately in sterile PCR tubes
7. Ladder was added to well no 4 and the other wells were loaded with the amplified DNA in order on either side of the marker.
8. Electrodes were connected and run at 100v for 45 minutes until the loading dye was seen at 3/4th of the gel.
9. The trough was removed and gel was viewed under UV illuminator for the presence of bands.

2. **DNA sequencing**

a. Sample preparation

DNA from the culture was amplified to obtain a volume of 100µl. This was purified by following the steps in the PCR purification kit (GENEI).

b. DNA Sequencing was done for the amplified products (Macrogen.Inc)

Results

Out of the 117 respiratory samples collected for the identification and drug susceptibility testing of *Mycobacterium tuberculosis*, 104 (88%) were sputum, 11(9%) were bronchoalveolar lavage, and 2 (2%) were pleural fluid as shown in Fig 1.

The RNTCP sputum grading ranged from scanty to 3+. The distribution of various grades and the percentage of sputum samples for each grading are shown in Fig 2

Of the 117 specimens processed, 13 had no growth and 3 samples showed growth on Lowenstein Jensen medium with p-nitro benzoic acid (PNB). Out of the 104 positive cultures, 3 which were resistant to PNB were excluded.

The proportion of the males was more than the females in the 101 cases from whom cultures were positive and included in the study. The percentage of males and females was 76% and 24% respectively giving a ratio of 3:1 (Fig 3)

The age distribution shown in Fig4 indicates the preponderance of tuberculosis in the fourth and fifth decade of life with the next common occurrence being in the age groups 35 – 44 and 55 - 64. Most of the specimens were from new cases (80%) and only 20% comprised of old cases which included relapse and default cases

On the basis of a panel of 3 tests the isolates were identified and characterised as belonging to *M.tuberculosis* complex. Isolates of *Mycobacterium tuberculosis* complex are positive for Niacin and Nitrate tests with negative Catalase test. Niacin test was positive in 71% isolates, Nitrate reduction in 75% and Catalase was negative in 100% of isolates (Fig5).

Drug susceptibility test done by absolute concentration method for 2 drugs – Isoniazid and Rifampicin, shows that 94% of the isolates were sensitive to both the drugs. The presence of >20 colonies at the MIC of ≥ 0.2µg/ml for Isoniazid and at ≥ 32 µg/ml for Rifampicin was seen in 5 isolates and was categorised as Multi-drug resistant strain. There were 5 more isolates found to be resistant to Isoniazid alone and one isolate was resistant to Rifampicin only. Of the 5 MDR strains, four isolates were from old cases and one was from a new case.

A total of 70 clinical isolates subjected for sensitivity testing by MTT assay showed the following results Table 4

The resistance pattern by MTT assay showed results similar to the absolute concentration method. The number of MDR isolates were 5 by both methods but there was a slight difference in the mono-resistant pattern in both the drugs. Excluding the MDR strains 5 and 1 isolates were found to be resistant to Isoniazid and Rifampicin respectively by absolute concentration method and the numbers were 4 and 2 for Isoniazid and Rifampicin by MTT assay.(Table 5). The sensitivity of the MTT assay was 90% for Isoniazid and 100% for Rifampicin

The results of DNA sequencing for resistant isolates done at Macrogen.Inc showed mutations in the codon 526 and codon 531(Fig 7).

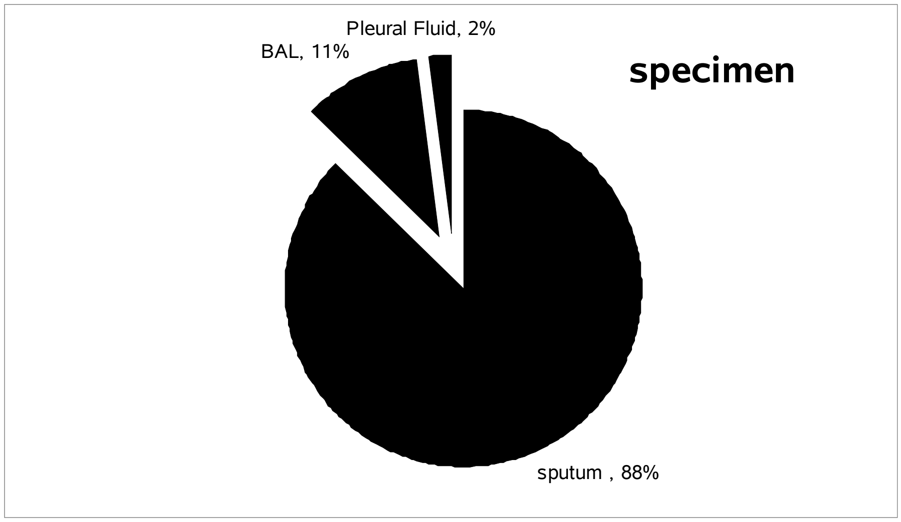


Fig 1: Percentage of various specimens collected for the study.
BAL – bronchoalveolar lavage

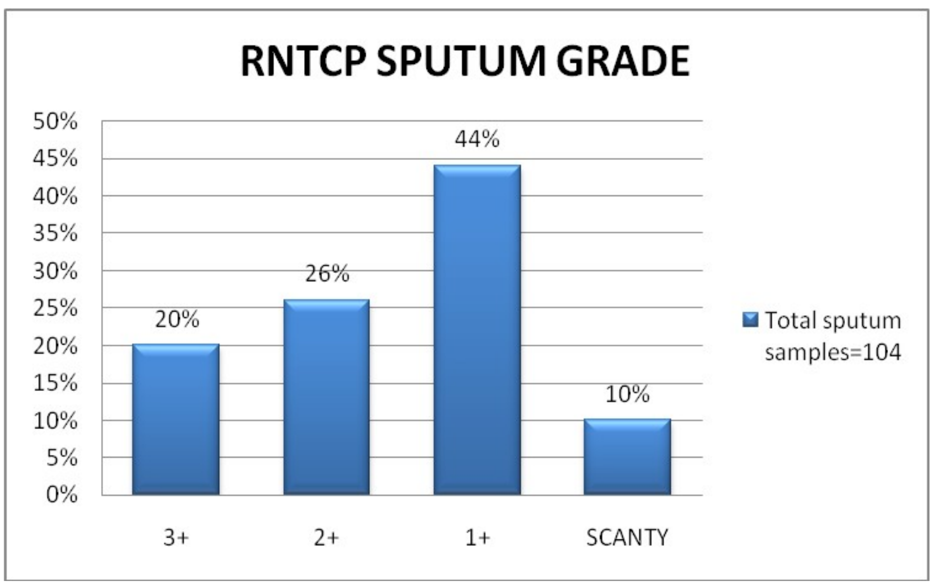


Fig 2: Percentage of sputum samples in various RNTCP grades.

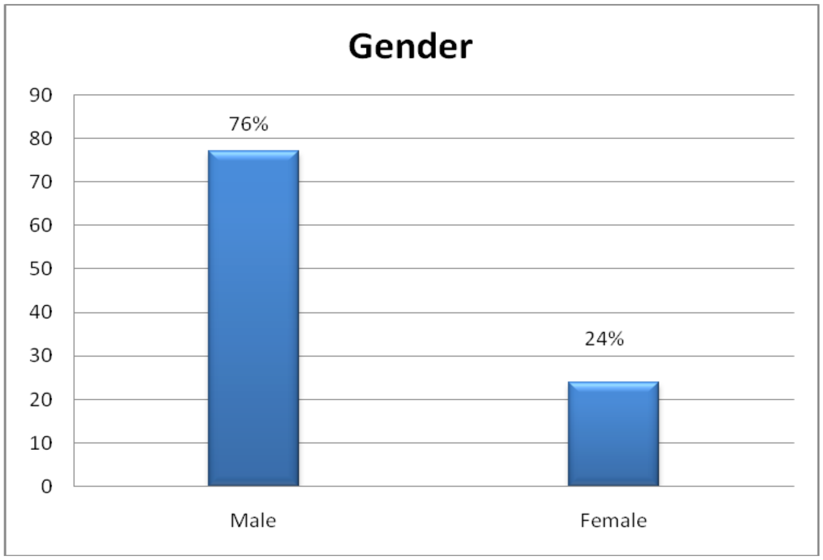


Fig 3: Percentage of gender distribution in the study group.

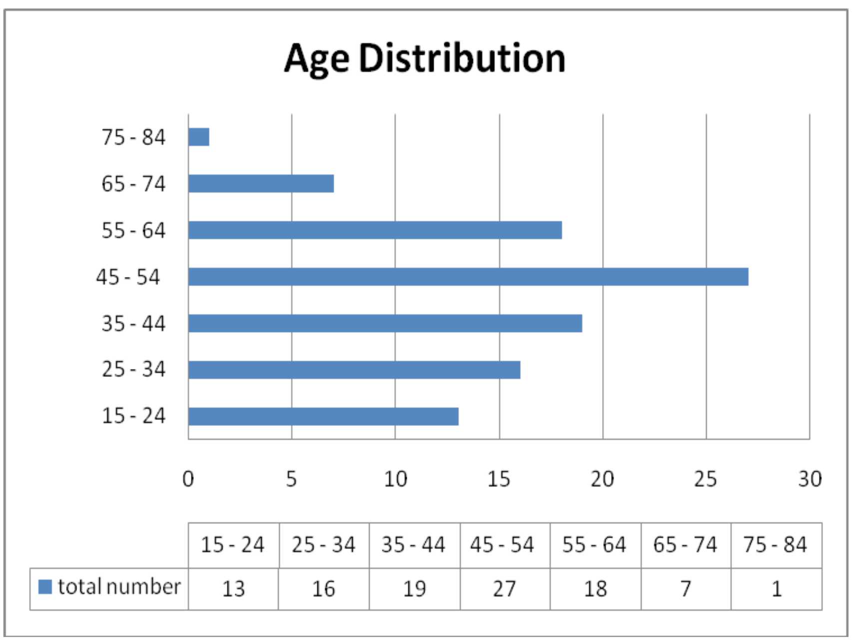


Fig 4: Age distribution among the study group

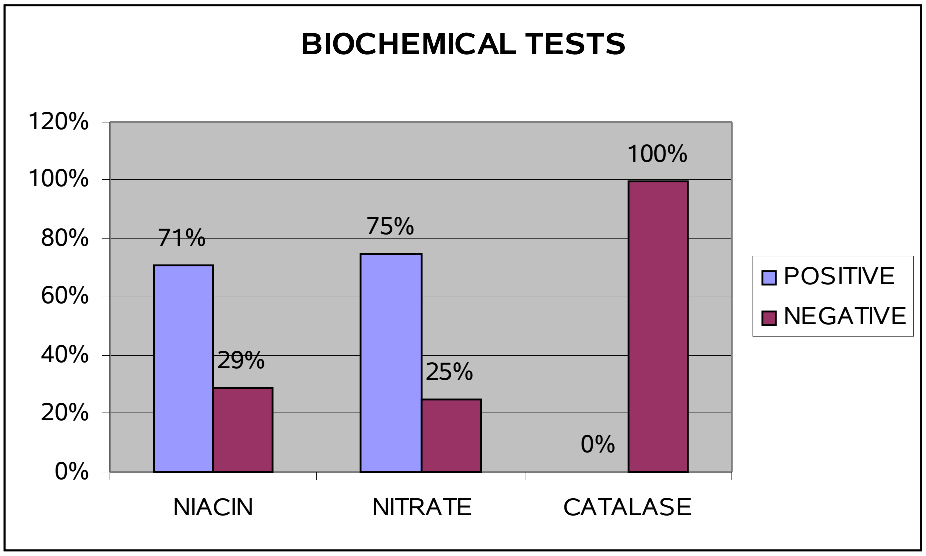


Fig 5: Percentage of positive and negative results of the biochemical tests

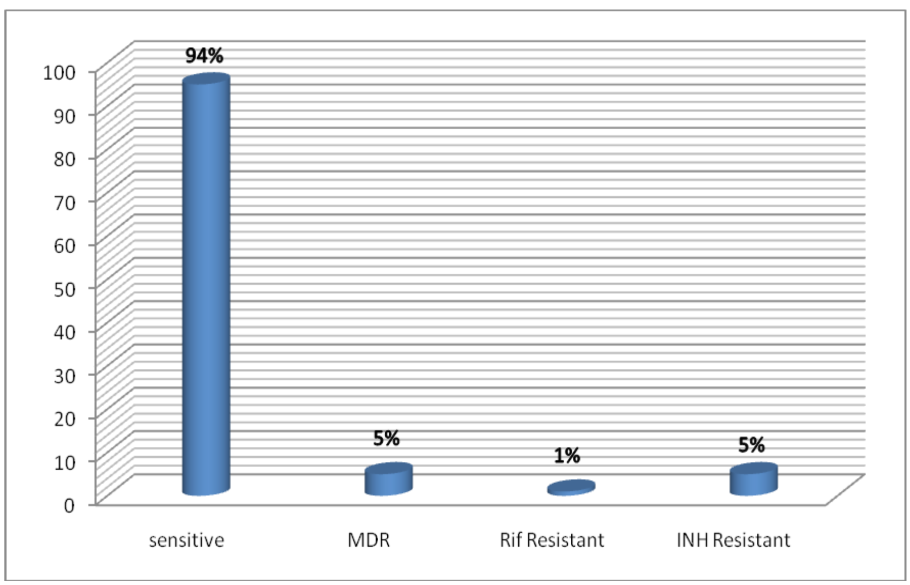


Fig 6: Susceptibility pattern of the *M.tuberculosis* isolates in the study

MDR – Multidrug resistant, Rif – Rifampicin, INH - Isoniazid

Table 4: Comparison of resistance pattern by absolute concentration method and MTT assay

COMPARISON OF SUSCEPTIBILTIY TESTS	Isoniazid				Rifampicin			
	Absolute concentration method 0.2µg/mL		MTT assay 0.4µg/mL		Absolute concentration method 32µg/mL		MTT assay 2µg/mL	
	R	**S**	**R**	**S**	**R**	**S**	**R**	**S**
	10	**60**	**9**	**61**	**6**	**64**	**7**	**63**
TOTAL	**70**		**70**		**70**		**70**	

R – resistant S - sensitive

Table 5: Comparative evaluation of the resistant isolates from both susceptibilty tests

Drug Susceptibiltiy Test	MDR	INH mono resistant	Rifampicin mono resistant
Absolute concentration method	5	5	1
MTT assay	5	4	2

MDR – multidrug resistant , INH – Isoniazid

H37Rv

507 508 509 510 511 512 513 514 515 516517 518 519 520 521522 523 524 525 526 527 528 529 530 531 532 533
GGCACCAGCCAGCTGAGC CAATTC ATGGACCAGAACAACCCGCTGTCGGGGTTG ACCCAC AAGCGCCGA CTGTCGGCGCTG

Gly Thr Scr Gln Leu ser Gln Phe Met Asp Gln Asn Asn Pro Leu Ser Gly Leu Acc His Lys Arg Arg Leu Ser Ala Leu

Rifampicin Resistant Strains

507 508 509 510 511 512 513 514 515 516517 518 519 520 521522 523 524 525 526 527 528 529 530 531 532 533
GGCACCAGCCAGCTGAGC CAATTC ATGGACCAGAACAACCCGCTGTCGGGGTTG ACC GAC AAGCGCCGA CTGTCGGCGCTG

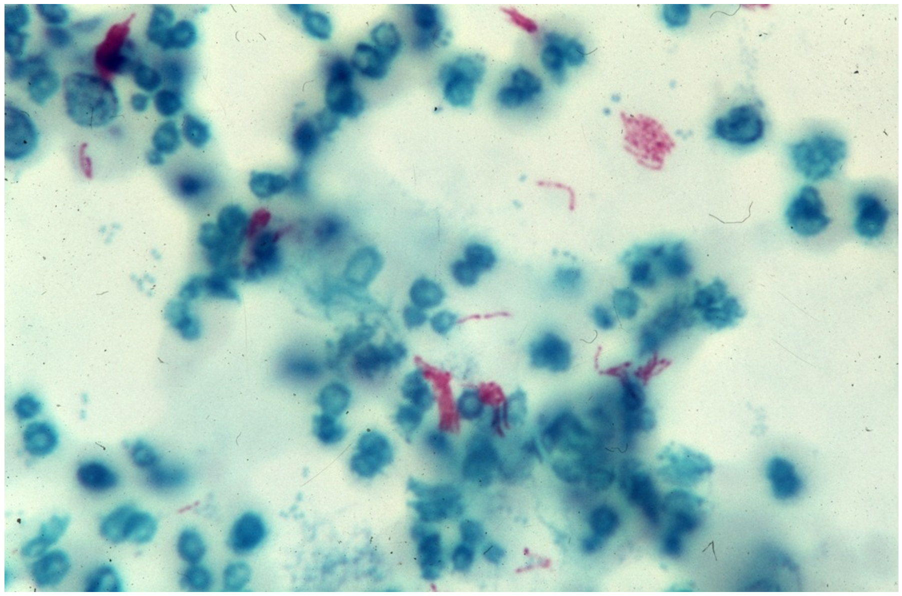
Gly Thr Scr Gln Leu ser Gln Phe Met Asp Gln Asn Asn Pro Leu Ser Gly Leu Acc **Asp** Lys Arg Arg Leu Ser Ala Leu

507 508 509 510 511 512 513 514 515 516517 518 519 520 521522 523 524 525 526 527 528 529 530 531 532 533
GGCACCAGCCAGCTGAGC CAATTC ATGGACCAGAACAACCCGCTGTCGGGGTTG ACCGAC AAGCGCCGA CTG TTG GCGCTG

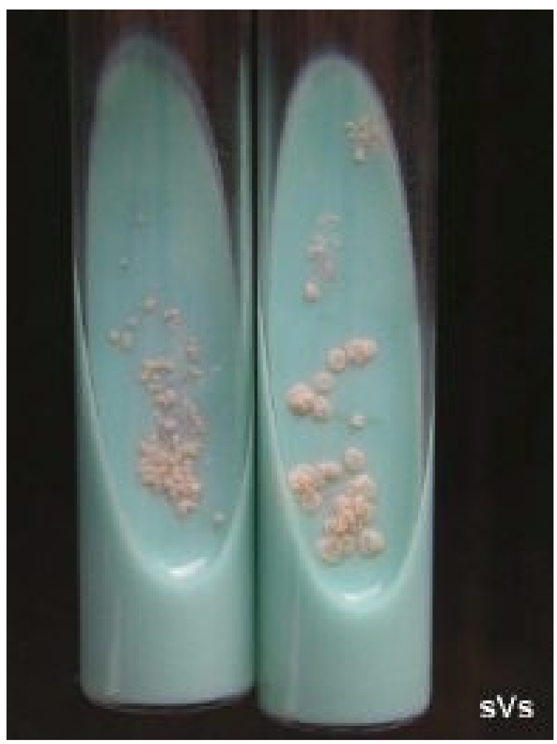
Gly Thr Scr Gln Leu ser Gln Phe Met Asp Gln Asn Asn Pro Leu Ser Gly Leu Acc Asp Lys Arg Arg Leu **Leu** Ala Leu

Fig 7: The rpoB gene (81bp region) from standard strain H37Rv and Rifampicin resistant strains.

COLOUR PLATE I

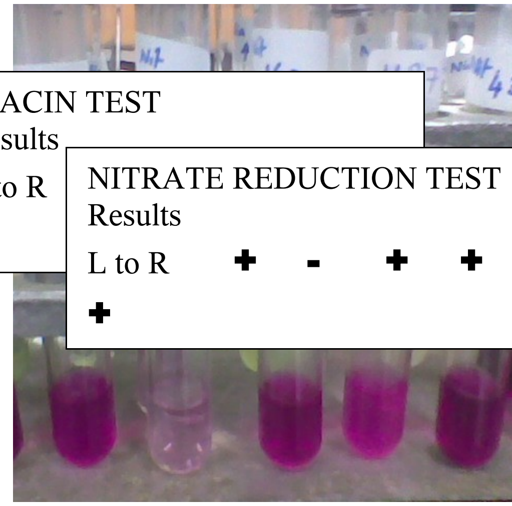


A: Ziehl Neelsen staining of smear showing acid fast bacilli (grade 3+)



B: Lowenstein Jensen Media showing colonies of *M.tuberculosis*

COLOUR PLATE II
BIOCHEMICAL TESTS FOR IDENTIFICATION



NIACIN TEST
Results
L to R
✚

NITRATE REDUCTION TEST
Results
L to R ✚ - ✚ ✚
✚

HEAT STABLE CATALASE TEST
Results
L to R - ✚ - - -

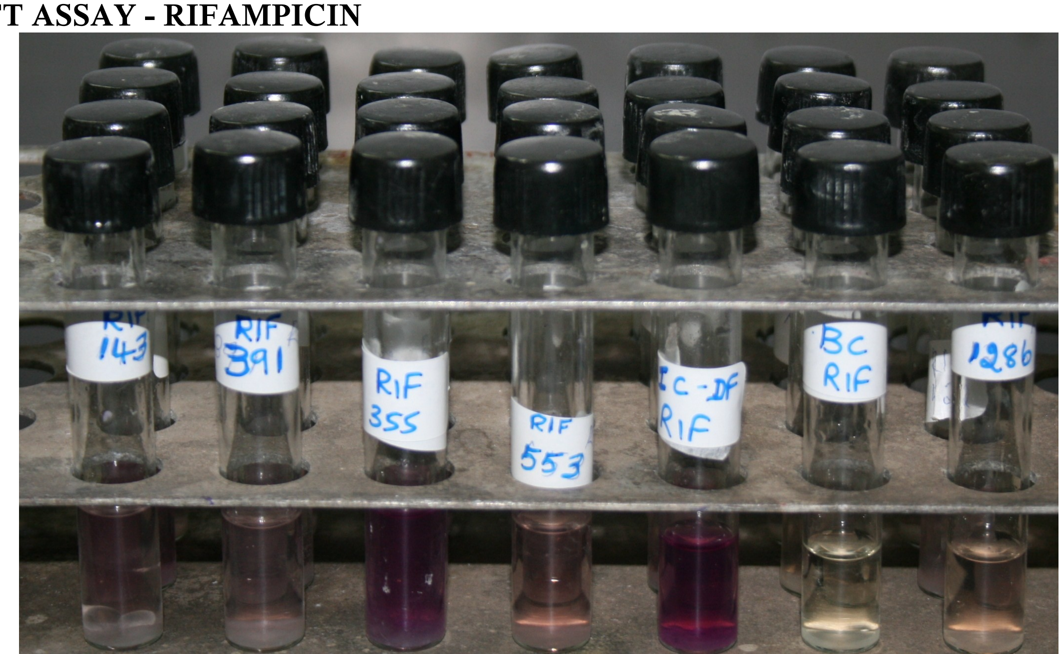
COLOUR PLATE III
ABSOLUTE CONCENTRATION METHOD

Picture showing multi-drug resistant strain

L J **L J-PNB** **0.2** **1** **5** **32** **64** **128**

Drug
INH (µg/ml) **RIFAMPICIN (µg/ml)**

MTT ASSAY - RIFAMPICIN

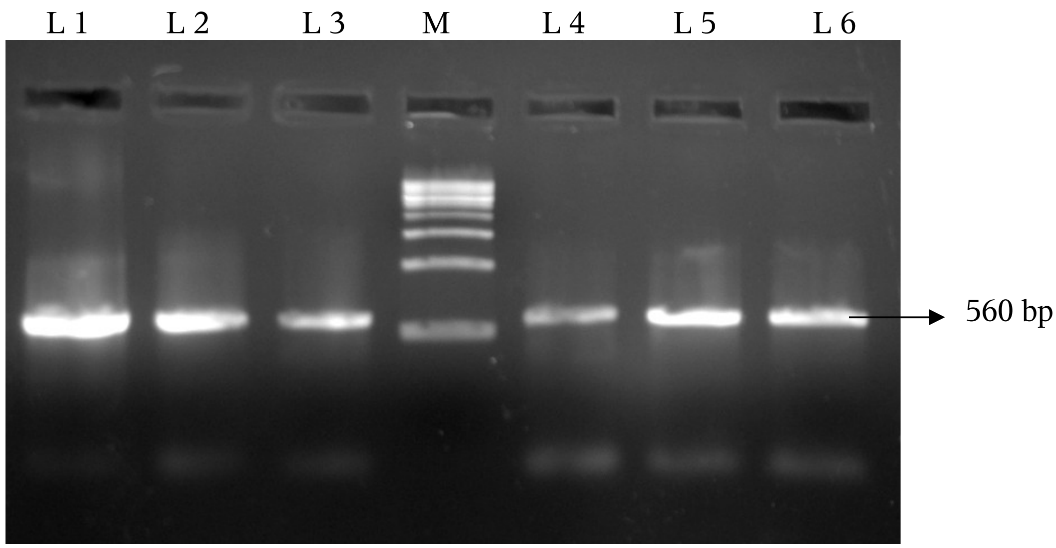


S **S** **R** **S** **R** **S** **S**
S – SENSITIVE **R – RESISTANT**

COLOUR PLATE IV

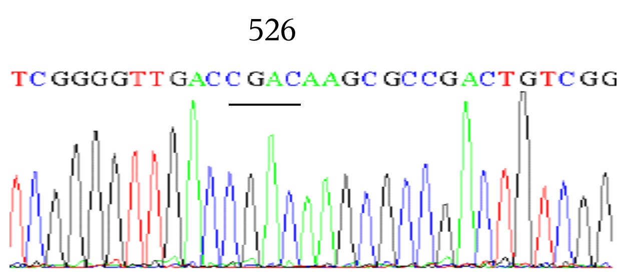
Gel electrophoresis of the amplified DNA from

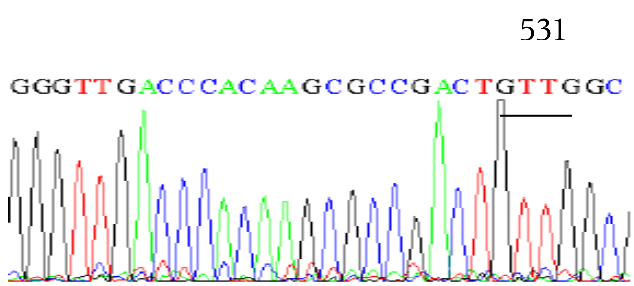
Rifampicin resistant isolates



L 1 – MDR 1, L 2 – MDR 2, L 3 – MDR 3, M – 500 bp marker
L 4 – MDR 4, L 5 – MDR 5 , L 6 – RIF mono resistant

RESISTANT STRAINS





Discussion

The current global concern in the treatment of tuberculosis (TB) is the emergence of resistance to the two most potent drugs *viz*., Isoniazid and Rifampicin[25]

The magnitude of drug resistance prevalent in a community can have significant implications for the outcome of a tuberculosis programme, because patients with drug-resistant bacilli respond much less favourably than those with sensitive bacilli. Also, 'failure' of treatment cases in both the groups would infect the non-infected with drug-resistant bacilli and render treatment ineffective in them[45].

During the early development of drug resistance only a small proportion of resistant bacteria are present within a milieu of sensitive bacteria. In a developing country like India with poor patient compliance, inappropriate and/or inadequate treatment leads to emergence of drug resistant strains by selective multiplication of resistant mutants within the lesions, in spite of the presence of growth-inhibitory concentrations of a drug[46]. It is important to monitor the level of drug resistance in the community, and adopt appropriate treatment regimens.

The identification of Mycobacterium to the species level plays an important role in providing adequate patient management. Hence we characterized our isolates as *M.tubeculosis* complex by the following tests – Niacin production, Nitrate reduction, Heat stable catalase at 68ºC. Classical culture and biochemical tests, when properly applied, detect M. tuberculosis in clinical samples, with reasonable sensitivity[47]. This has been supported by similar studies which have identification of M.tuberculosis by biochemical tests and molecular methods[47-49]. In a study by Kothadia et al[50], 16% of the isolates were found to be Niacin negative *M.tuberculosis* whereas we encountered only 4% of such isolates.

In this study 100% of the isolates were sensitive to p-nitrobenzoic acid and hence was used as a reliable test along with the other biochemical tests for speciation. This correlates well with similar studies done earlier[51,52].

Though the proportion method is the gold standard for drug susceptibility testing as per the NCCLS guidelines[53], absolute concentration method was followed due to its technical simplicity for inoculum preparation and for reading the results[46]. According to Heifets 2000[54], the absolute concentration method can be used when the critical drug concentration can be standardized in a laboratory. The Absolute concentration method was standardized in our laboratory with a standard MDR and sensitive strain (H37Rv)

However because the organism is slow growing, laboratory diagnosis by these methods can take as along as eight to ten weeks. Rising numbers of resistant and MDR-TB strains have led to the pressing demand for a rapid, appropriate and accurate DST.

The MTT assay is a rapid colorimetric assay giving results in 2 weeks, compared with the conventional absolute concentration method, which takes 4 weeks before the results can be obtained. In our study this assay showed good sensitivity for both Isoniazid (90%) and Rifampicin (100%) There were high concordance levels for Rifampicin and sufficiently high levels for detecting resistance for INH in our study as compared with other studies which showed sensitivity of 100% and 96% for Rifampicin and INH respectively[37,38,43,55]

According to another study, MTT assay can also be applied to direct sputum samples for detection of Rifampicin resistance and when compared with agar based method showed a 98.5% match giving rapid results in 2 weeks[56].

Resistance could be primary, i.e., infection from a source with resistant bacilli, or acquired due to inappropriate drug prescription, irregular drug supply to patients or non-compliance on the part of patients. The accuracy of classification depends on the efficiency with which history of previous treatment is elicited which is often poor in developing countries[45].

A review of the few authentic reports from India[57-61] indicate that there is no clear evidence of an increase in the prevalence of initial resistance over the years but there is a higher prevalence of acquired resistance reported from several regions, though based on smaller numbers of patients[25].

In India the primary resistance to both the drugs range from 0 – 3.4% in different studies which is comparable to our data (1%). Our data on primary MDR –TB was also consistent with the global median prevalence of 1.1% (range 0 – 14.2%)[62]

The global resistance scenario covering 42% world's population for acquired MDR-TB showed a median prevalence of 7% with a range of 0 – 58.3% which places our data of acquired resistance (4%) well within the global range. Our study data when compared with the available studies in India[57,63,64] and Tamil Nadu[77,78,87] was lower. This may be due to our study representing only a proportion of patients attending the hospital.

Analysis of almost 90,000 strains between 1994 and 2002 from countries globally confirmed that, more strains were resistant to INH than to any other drug (0-42%)[25,66]. Our study data showed a similar response of higher resistance to INH (10%) compared to 6% resistance in Rifampicin

While host genetic factors may probably contribute, incomplete and inadequate treatment is the most important factor leading to the development of MDR-TB[5,67].

In our study group, the ratio of male to female of 3:1 was in accordance with other studies in India which also showed a male preponderance of 1.4-5:1[68,69].

Both prevalence and incidence rise with age, in both sexes. In surveys conducted so far in the country[69] there was a preponderance of tuberculosis in the 4th and 5th decade of life which is similar to our data. A slight variation was seen in a recent study by Chakraborty ,2004[70] where in the peak concentration was seen at 25-34 yr age group

Rifampin (RIF) resistance serves as a surrogate marker for the detection of MDR TB, as 90% of Rifr isolates are also isoniazid resistant. The genetic basis for RIF resistance in approximately 95% of the cases is due to mutations in an 81-bp RIF resistance-determining region (RRDR) of the *rpoB* gene, corresponding to codons 507 to 533 (*Escherichia coli* numbering system), which codes for the beta subunit of the RNA polymerase of *M. tuberculosis*.

Different groups of workers from diverse regions of the world, have thus far reported around 65 substitutions, 12 deletions, and 4 insertions in the RRDR of the *rpoB* gene.

Determination of the mutation patterns among large numbers of isolates from different parts of India is essential, for rapid detection and also identification of any hot-spot regions in the country for proper implementation of TB control programs. . Moreover, it is well known that clinical isolates from southern India are very different from isolates from other parts of the world. It is of interest to determine whether these southern Indian isolates show any different kinds of mutations in the RRDR region of the *rpoB* gene[18].

In our study, sequencing showed mutations in the commonly occurring codons 531 (TCG to TTG) and 526 (CAC to GAC) of the rpoB gene. Previous studies have reported a wide range of frequencies for mutations at codon 531 (20 to 71%) and codon 526 (0 to 30%)[71,72,73]. Hirano et al[74] has reported mutations in Indian isolates at codon 531 (TCG to TGG) and codon 526 (CAC to GAC).

To conclude, in our study we have detected MDR in 5% of the study group and mutaions were seen in commonly occurring codons. No novel mutations were encountered.

Summary

- The study was taken up mainly to find out the Multi drug resistance in *Mycobacterium tuberculosis* in this part of Tamil Nadu
- A total of 117 respiratory specimens were collected from the RNTCP outpatient clinic and in-patients from PSG hospitals.
- RNTCP criteria was followed for sample collection , staining and grading of sputum smears
- Among the samples, sputum grading ranged from scanty, 1+, 2+ and 3+
- Out of 117 specimens collected, 104 (89%) were culture positive and 3 more isolates were excluded from the study as they were resistant to p-nitro benzoic acid suggesting Non-tuberculous mycobacteria
- A total of 101 culture positive isolates were further subjected for characterization using biochemical tests namely Niacin production, Nitrate reduction and Heat stable catalase.
- Among the study group there was male preponderance with a male to female ratio of 3:1
- The peak age distribution was found to be 45 – 54 years (27%)
- All the 101 isolates were found to belong to *M.tuberculosis* complex of which 25% were found to be *M.bovis*

- Drug susceptibility testing was done by Absolute concentration method on all the above isolates

- The percentage of multi-drug resistance in old cases and new cases were 4% (acquired resistance) and 1% (primary resistance) respectively.

- Mono-resistance to INH was 4% in new cases and 1% in old cases whereas Rifampicin mono-resistance of 1% was seen in one new case only

- MTT ((3-4,5-dimethylthiazol-2-yl-2,5-diphenyl tetrazolium bromide) assay done for 70 isolates of *M.tuberculosis* from sputum samples showed a sensitivity of 90% and 100% for INH and Rifampicin respectively. Results of MTT assay are in concordance with those obtained by Absolute concentration method.

- For the six isolates (5MDR and 1 Rifampicin mono- resistant) , DNA was extracted by CTAB (Cetyl trimethylammonium bromide) – Nacl method and amplified (560bp) with specific primers for rpoB gene targeting the 81bp hotspot region using Eppendorf thermocycler

- The primers used were

Forward primer	5’ GGTGGAAACCGACGACATCGA 3’
Reverse primer	5’ CGCATCGATCGGCGAATTGG 3’

- Sequencing done for the above amplified products (Macrogen. Inc) showed mutations at codon 531 and codon 526

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Appendix

Preparation of Staining Solution

1. **Carbol Fuchsin**

Basic fuchsin (Hi media)	10 g
Absolute alcohol	100 ml.
Phenol	50 g.
Distilled water	900 ml.

- Weigh 5 gm of basic fuchsin dye & transfer it to a 250 ml conical flask.
- Add 50 ml of absolute alcohol & dissolve the dye by placing it in a water bath at 60º C. Avoid direct heating.
- Place the phenol bottle in the water bath at 80º C for melting
- Measure 50 ml of Phenol and add to the basic fuchsin solution and mix gently
- Transfer the contents into a 1L measuring cylinder
- Add 900 ml of distilled water to make up the final volume to 1000 ml.
- Pour the solution through filter paper (Whatman No 1) and store filtered solution in a glass bottle. Label the bottle as 1% Carbol Fuchsin and the date of preparation.

2. **25% Sulphuric acid**

Sulphuric acid, A.R.	25 ml
Distilled water	75 ml

(Note: Always add the acid to the water with mixing and never vice versa)

- Take 75 ml distilled water in a flask
- Carefully add concentrated sulphuric acid to the water

- Mix gently and store it in an amber coloured bottle and label it as 25% sulphuric acid and date of preparation.

3. **0.1% Methylene Blue**

Methylene blue (BDH)	-	0.5 g
Distilled water	-	500 ml

- Weigh 0.5 gm of methylene blue and transfer it to a 1L conical flask
- Add 500 ml of distilled water
- Shake well & dissolve
- Store in a glass bottle with the label as 0.1% Methylene blue and write the date of preparation

4. **Sodium Hydroxide Solution 4%**

Sodium hydroxide Pellets, A.R.	- 40 g
Distilled water	- 1000 ml

- Weigh 40 gm of Sodium hydroxide and transfer to a I litre flask
- Add 1000 ml of distilled water to it and mix thoroughly until it dissolves.
- Distribute in 100 ml aliquots into 250 ml conical flasks and cover the mouth of the flask with cotton bunk. Wrap with aluminium foil.
- Autoclave at 15lbs/20 minutes, cool and store at 37ºC until use.

Preparation of Media

5. **LOWENSTEIN JENSEN MEDIUM**

Mineral Salt-Malachite green solution (SSMG)

Potassium dihydrogen orthophosphate, AR, KH2PO4 - 14.4g.

(0.4%)

Magnesium sulphate, AR,(anhydrous)(0.4%)	- 1.44 g.
Magnesium citrate (0.1%)	- 3.6 g
L-Asparagine, AR(0.6%)	- 21.6 g.
Glycerol, AR (2%)	- 72 ml
* Malachite green 2% solution	- 120 ml
Distilled water to	- 3600 ml.

*Malachite green solution 2%

Malachite green dye	25g
Distilled water	1250ml

Homogenization of eggs

- Select fresh hens' eggs not older than 7 days for the preparation of egg fluid.
- Cleanse eggs with soap water, place in a basin and wash in running water until the water is clear, then rinse in distilled water. Finally immerse in 70% alcohol for 5 minutes and leave to dry on a clean towel.
- Break the eggs individually into a beaker, and if fresh break the yolk with a sterile 10 ml pipette. Transfer the egg fluid into a 2 litre round flat-bottomed flask.
- The egg fluid is homogenized using a mechanical blender.
- Filter the egg fluid using sterile gauze and funnel

Preparation of L-J medium

- Measure out one litre of egg fluid using a sterile measuring cylinder and transfer into a 3 or 5 litre conical flask
- 600 ml of the sterilized salt solution with malachite green (SSMG) is transferred to the egg fluid gently. Shake the flask to mix thoroughly.

- Distribute approximately 10 ml of medium in 1 oz. universal containers (McCartney bottle).

Inspissation of media

- Pour distilled water into the inspissator tank through the side opening up to the mark. Switch on the inspissator and check the temperature to read 85ºC
- Place the bottles in a slanted position in the inspissator, cover the slopes with a quilt and coagulate the medium for 50 minutes at 85ºC.
- After 50 minutes remove the bottles from the insipissator and leave at room temperature.
- The inspissator temperature is noted periodically (every 15 mts).
- Re-inspissate the bottles at 85ºC for 30 minutes on the next day.
- Label the media tray with batch number and date of preparation.

Sterility check

After inspissation, the whole media batch should be incubated at 37ºC for 24 hours. The media bottles are randomly checked for contamination

Preparation of reagents for biochemical tests

6. NIACIN TEST

- 4% Aniline in alcohol

Measure 0.4 ml of aniline into 9.6 ml of absolute alcohol. This solution should be prepared fresh just before every batch of tests and should be colourless

- Cyanogen bromide solution, approx. 10%.

1gm of cyanogen bromide was weighed inside the Biosafety Cabinet (BSC) and added to 10ml of distilled water in a screw capped bottle. This solution is prepared fresh just before each batch of tests

7. **CATALASE TEST at 68^{0}C/pH 7.0**

a. 0.067M (M/15) phosphate buffer solution, pH 7.0

Na2HPO4, anhydrous 9.47 g

Distilled water 1 litre

Dissolve 9.47 g of disodium phosphate in 1 litre distilled water to provide 0.067 M solution (Solution 1).

KH2PO4 9.07 g

Distilled water 1 litre

Dissolve 9.07 g of dibasic potassium phosphate in 1 litre of distilled water to give 0.067 M KH2PO4 solution (Solution 2)

Mix 61.1 ml of solution 1 with 38.9 ml of solution 2. Check p^H.

b. Hydrogen peroxide 30% solution.

Analytical grade H2O2 is 30%. Store in the refrigerator.

c. Tween-80, 10%

Tween-80 10 ml

Distilled water 90 ml

Mix Tween-80 with distilled water and autoclave at 121oC for 10 minutes. Allow to cool. Store in the refrigerator.

8. Nitrate Reduction Test

(a) Substrate: 0.1M sodium nitrate

Sodium nitrate	0.85 g
M/45 phosphate buffer, pH 7.0	100 ml

Prepare M/45 buffer by diluting M/15 buffer (as for 68oC catalase) 1:2 with distilled water. Dissolve the sodium nitrate in the buffer and distribute in 2-ml amounts in screw-capped test tubes. Sterilize at 15 lbs pressure for 15 minutes and store in the cold.

(b) Hydrochloric acid, 1:1

Hydrochloric acid, concentrated	10 ml
Distilled water	10 ml

Mix equal volumes of concentrated HCl and distilled water. Always add acid to water and not vice versa. Store at room temperature in a polythene bottle.

(c) Sulphanilamide, 0.2%

Sulphanilamide	0.2 g
Distilled water	100 ml

Dissolve 200 mg sulphanilamide in 100 ml distilled water Store in an amber colored bottle in the refrigerator.

(d) Coupling agent

N- (1-naphthyl)-ethylene diamine di-HCl	0.1 g
Distilled water	100 ml

Dissolve 100 mg of the coupling agent in 100 ml distilled water. Store in a dark bottle in the refrigerator.

(e) Zinc dust.

9. L-J MEDIUM CONTAINING *p*-NITRO BENZOIC ACID (PNB)

Stock Solution

- Weigh 0.5 gm p-nitrobenzoic acid and transfer it into a concial flask containing 20 ml of dimethyl formamide. Mix gently to dissolve the PNB.
- Transfer the entire quantity to 1 litre of L-J fluid giving a final concentration of 500 µg/ml.
- Label the cap of the universal container as PNB
- Mix thoroughly, distribute approximately 10ml volume in the labeled universal container and inspissate.

Sterility check

- After inspissation, the whole media batch should be incubated at 37° C for 24 hours. The media bottles are randomly checked for contamination

10. PREPARATION OF DRUG MEDIA FOR SENSITIVITY TESTS

ISONIAZID

Stock solution

- Weigh accurately 100 mg of Isoniazid using butter paper and electronic balance.
- Transfer the weighed drug into a sterile McCartney bottle
- Add 10 ml of double sterile distilled water (DSDW) to the McCartney bottle
- Shake well to dissolve the drug completely.
- The final concentration of the stock solution is 10,000 mg/l.
- Filter the solution using a membrane filter

Drug Susceptibility Test for 12 isolates

Stock (10,000 µg/ml)	D.water	Total (ml)	Working solution - concentration	Working solution	LJ (ml)	Final conc in LJ (µg/ml)
0.1 ml	4.9ml	5 ml	200 µg/ml	3ml	120 ml	5 µg/ml
0.1 ml	24.9 ml	25 ml	40 µg/ml	3ml	120 ml	1 µg/ml

Stock 40 µg/ml	D.water	Total (ml)	Working solution concentration	Working solution	LJ (ml)	Final conc in LJ (µg/ml)
1 ml	4 ml	5 ml	8 µg/ml	3 ml	120 ml	0.2 µg/ml
0.5 ml	4.5 ml	5 ml	4 µg/ml	0.6 ml	24 ml	0.1 µg/ml
0.1 ml	1.9 ml	2 ml	2 µg/ml	0.6 ml	24 ml	0.05 µg/ml

Stock 2 µg/ml	D.water	Total (ml)	Working solution concentration	Working solution	LJ (ml)	Final conc in LJ (µg/ml)
0.5 ml	0.5	1 ml	1 µg/ml	0.6	24	0.025 µg/ml

RIFAMPICIN (RIF)

Stock solution

- Weigh accurately 100 mg of rifampicin powder using butter paper and electronic balance and transfer the drug into a sterile McCartney bottle
- Add 10 ml of dimethyl formamide to the drug and shake well to dissolve.
- The final concentration of the stock solution is 10,000 mg/l.
- The solution is self sterilizing.

Drug Susceptibility Test for 12 isolates

Stock	D.water	Total (ml)	Working solution concentration	Working solution	LJ (ml)	Final conc in LJ (µg/ml)
10,000 µg/ml	-	-	-	1.540 ml (undiluted)	120 ml	128 µg/ml
10,000 µg/ml	-	-	-	770µl (undiluted)	120 ml	64 µg/ml
0.4 ml (400µl)	1.6 ml	2 ml	2000 µg/ml	1.920 ml	120 ml	32 µg/ml
0.2 ml (200 µl)	9.8 ml	10 ml	200 µg/ml	1.920 ml	24 ml	16 µg/ml
-	-	-	"	960 µl	24 ml	8 µg/ml
-	-	-	"	480 µl	24 ml	4 µg/ml

Distribute using pourers and inspissate once at 85°C for 50 minutes. Label the tray with the batch no., date of preparation and store in the cold room.

11. MTT ASSAY

Test Medium Used

Middle brook 7H9 broth supplemented 10% OADC and 0.01% glycerol.

Media Composition

Middle brook 7H9 broth base (Himedia): (pH - 6.6+/-0.2)

Composition per liter:

Na_2HPO_4.. 2.5g

KH_2PO_4..1.0g

Monosodium glutamate..0.5g

$(NH_4)_2SO_4$..0.5g

Sodium citrate..0.1g

$MgSO_4 \cdot 7H_2O$...0.05g

Ferric ammonium citrate...................................0.04g

$CuSO_4 \cdot 5H_2O$..1.0mg

Pyridoxine..1.0mg

$ZnSO_4 \cdot 7H_2O$...1.0mg

Biotin..0.5mg

$CaCl_2 \cdot 2H_2O$..0.5mg

Middle brook OADC enrichment............... ….100 mL

Glycerol..2 mL

Middle brooks OADC Enrichment (HIMEDIA):

Composition per 100.0mL:

Bovine albumin fraction V.................................5.0g

Glucose..2.0g

NaCl...0.85g

Oleic acid...0.05g

Catalase...4.0mg

Preparation of Middle brooks OADC Enrichment:

The above mentioned components were added to distilled/deionized water and the volume brought upto 100mL. It was then mixed thoroughly. Filtered and sterilized.

Preparation of Medium: Glycerol was added to 900mL of distilled/deionized water and the remaining media components are also added, except Middle brook OADC enrichment.

- It was then mixed thoroughly. Gently heated and brought to boil.
- The solution was then autoclaved at 121°C for 15 min with 15 psi pressure. It was cooled to 50°–55°C.
- 100mL of sterile Middle brook OADC enrichment was added aseptically and mixed thoroughly.
- 4 ml was distributed into sterile tubes or flasks. The sterility checking was done on blood agar to rule out contamination.

Preparation of Inoculum

- Several loopful of growth from 4 week old cultures on LJ medium were transferred to sterile tubes with glass beads containing 4 ml of Middle brook 7H9 broth.
- Tubes were then vortexed for five minutes to breakup larger clumps. Suspension was then allowed to stand for 10 minutes before transfer of the supernatant to another sterile tube.
- The supernatant fluid was again allowed to stand for 15 minutes before it was transferred to a third sterile tube.
- The suspension was then adjusted to turbidity equivalent to No.1 McFarland's standard using sterile saline.
- This standard suspension was used for rapidly screening drug susceptibility of Mycobacterium tuberculosis strains using MTT assay.

Preparation of McFarland's Standard

- 1% barium chloride was prepared by dissolving 1g of barium chloride in 100ml of sterile distilled water.

- 1% sulphuric acid was prepared by dissolving 1 ml of sulphuric acid in 99 ml of sterile water.

- For 0.5 McFarland's standard, 10ml of 1% sulphuric acid was taken in a test tube and 0.05 ml of 1% barium chloride was added by removal of 0.05 ml of sulphuric acid from the tube.

- Similarly for 1 McFarland's standard, 10 ml of 1% sulphuric acid was taken in a test tube and 0.1 ml of 1% barium chloride was added by removal of 0.1 ml of 1% sulphuric acid from the test tube.

Preparation of Drug Solution

Preparation of Stock Solutions & Working Concentrations

Rifampicin (Obtained from SIGMA)

Stock solutions of Rifampicin were prepared as 20 mg/mL in dimethylsulfoxoide (DMSO- Obtained from QUALIGENS FINE CHEMICALS) and were dispensed in 0.1mL aliquots. They were stored at −70°C until further use.

The working concentrations were prepared subsequently by diluting with 7H9 broth supplemented with OADC. 5µl of Rifampicin from stock was added to 50 ml of Middle brook 7H9 broth to achieve the concentration of 2µg/ml.

Isoniazid (Obtained from SIGMA)

Stock solutions of Isoniazid were prepared 10 mg/mL in sterile distilled water (DW) and were dispensed in 0.1mL aliquots. They were stored at −70°C until further use.

The working concentrations were prepared subsequently by diluting with 7H9 broth supplemented with OADC. 2 µl of Isoniazid from stock was added to 50 ml of Middle brook 7H9 broth to achieve the concentration of 0.4µg/ml.

Preparation of MTT Solution

MTT (3-4, 5-dimethylthiazol-2-yl-2, 5-diphenyl tetrazolium bromide) obtained from SIGMA.

Contents

MTT-5 mg

Phosphate buffer saline (PBS) - 1 ml [pH 7.2]

Procedure

- 5 mg of MTT was added to 1ml of phosphate buffered saline.

Preparation of Phosphate Buffered Saline [pH 7.2]

Contents

Obtained from HIMEDIA

Sodium chloride (NaCl) - 0.9 g

Potassium dihydrogen phosphate (KH2PO4) - 0.317 g

Disodium hydrogen phosphate (Na2HPO4) - 0.181 g

Distilled water- 100 ml

Procedure

- The contents were weighed and added to distilled water.
- The pH was adjusted to 7.2 using sodium hydroxide (NaOH)
- The solution was sterilized by autoclaving.

Preparation of 0.1 N Hcl in Isopropyl alcohol

Contents is obtained from QUALIGENS FINE CHEMICALS

0.1 N Hcl - 365µl

Isopropyl alcohol – 100 ml

Procedure

- Hcl is added in Isopropyl alcohol and stored in tightly capped bottles.

12. Preparation of chemicals for DNA extraction

CTAB - NaCl Method:

REAGENTS REQUIRED

- TE buffer (pH 8)

  Stock (1M Tris, 0.5 M EDTA)

  Working (10mM Tris, 1mM EDTA)

- Lysozyme- 20 mg/ml in Milli Q water
- Sodium dodecyl sulphate-10% (10mg in 100ml milliQ water)
- Proteinase k- 10 mg/ml in milli Q water
- Sodium chloride (NaCl) -5M
- CTAB (Cetyl trimethylammonium bromide) -NaCl reagent (4.1g NaCl+ 80 ml distilled water+ 1G CTAB-made upto 100 ml)
- Chloroform : Iso amyl alcohol (24:1)
- Isopropanol

- 70% ethanol

MATERIALS REQUIRED

- refrigerated micro-centrifuge
- 1.5 ml Eppendorf tubes
- Micropipettes (100µl to 1 ml, 5µl to 50 µl)

TBE BUFFER

- Tris (89mM)
- Boric acid (89mM)
- EDTA (2mM)

ETHIDIUM BROMIDE – 10mg/ml in distilled water.

LOADING DYE

- Bromophenol blue – trace
- Sucrose – 40mg
- Distilled water – 1ml

Dissolve sucrose in distilled water and add a trace of bromophenol blue to it. Store the solution in refrigerator

Annexure